were stored at –70° until tested. Demographic data of patients such as age, sex, nationality, habitat and risk factors were also collected.

DNA Extraction and Sequencing

Viral RNA was extracted from 356 anti-HCV-positive serum specimens by using guanidine thiocyanate extraction [31]. Reverse transcription was performed using M-MLV reverse transcriptase (Promega, Medison, Wisc., USA). Partial core and NS5B regions were amplified by RT-PCR using specific primer pairs (core: primers 410/951 and 953/951, NS5B: primers NS5BF1/R1 and NS5BF2/R2) as previously described [32]. The resulting cDNA fragments were purified (HiYield Gel/PCR DNA Fragment Extraction Kit, RBC Bioscience, Taipei, Taiwan) and subjected to direct sequencing (First BASE Laboratories, Selangor, Malaysia). Nucleotide sequences were edited and assembled using Chromas LITE (v.2.0.1), BioEdit (v.5.09; Ibis Therapeutics, Carlsbad, Calif., USA) and SeqMan (DNASTAR, Madison, Wisc., USA).

HCV Genotyping

The genotype of each sample was determined by phylogenetic analysis of both the core and NS5B sequences. All sequences were aligned with reference genotypes retrieved from the Los Alamos HCV database [33] using CLUSTALW v1.83. Neighbor-joining trees of partial core and NS5B sequences were constructed using the Gojobori-Ishi-Nei 6-parameter method with bootstrap resampling tests using 1,000 replicates (http://clustalw.ddbj.nig.ac.jp). Sequences clustering on the same branch as the reference strain were assigned the reference genotype. Some of the samples with HCV genotype 6 had been previously published elsewhere [32]. The core region represents nucleotide positions 377–669 and the NS5B region represents nucleotide positions 8282–8591 relative to the sequence M62321.There were discrepant subtypes between the core and NS5B trees; therefore, we reanalyzed all sequences by using another method. All sequences were subjected to FindModel (http://hcv.lanl.gov/content/sequence/findmodel/findmodel.html) to find the best substitution model. Then, the HCV genotype classification was confirmed by constructing maximum likelihood trees with the GTR+Γ model and bootstrapped 1,000 times using MEGA version 5.05.

Phylogenetic Analysis of HCV Subtype 3a

In total, 136 core sequences and 132 NS5B sequences clustering in subtype 3a were obtained. To analyze subtype 3a of Thailand, the available subtype 3a sequences of the core and NS5B genes were retrieved from the Los Alamos HCV database and aligned with the respective subtype identified in this study [33]. Redundant sequences originating from the same individual or from an individual with unknown nationality were excluded as were those of insufficient length in nucleotide sequence. Multiple alignments were calculated using CLUSTALW v.1.83 and the phylogenic relationships of the core and NS5B were constructed using the neighbor-joining method based on the 6-parameter model with a bootstrap test performed on 1,000 replicates (http://clustalw.ddbj.nig.ac.jp). Particular clusters originating from Thai subtype 3a strains were investigated.

Evolutionary Analysis of HCV Subtype 3a

The HCV subtype 3a sequences of the partial core and NS5B regions originating from the same sample, which grouped in the Thai cluster, were selected for further analysis. This left 74 sequences of the NS5B dataset which was 325 nucleotides long (representing nucleotide positions 8286–8610 relative to the sequence M62321) and subjected to evolutionary analysis. The most appropriate nucleotide substitution model for the NS5B sequences dataset was determined using the jModeTest version 0.1. The best-fitting model of these data sets was GTR+Γ. HCV subtype 3a population dynamics were estimated using the strict molecular clock and the uncorrelated lognormal model under the substitution model mentioned above. Since the samples were not systemically collected and contained insufficient temporal data to directly estimate the evolutionary rate, the external rate was used in both the strict and relaxed clock model. The previously estimated substitution rate of 5.8×10^{-4} substitutions per site per year obtained from the NS5B gene was applied as a prior to generate an evolutionary timescale of years [34]. The most appropriate clock model was selected based on the Bayes factors computed by Tracer v.1.5. The bayesian skyline plot approach implemented in BEAST (Bayesian Evolutionary Analysis by Sampling Trees) v.1.5.4 (http://evolve.zoo.ox.ac.uk/beast/) was used to calculate the epidemic history. Each Markov chain Monte Carlo analysis was run for 50,000,000 states and sampling every 5,000 states. Markov chain Monte Carlo convergence, effective sample size, the date of the most common ancestor of the cluster and Bayes factors were computed and investigated using Tracer v1.5, and annotated phylogenetic trees were displayed by FigTree v. 1.2.2 (http://evolve.zoo.ox.ac.uk/beast/).

Nucleotide Accession Number

All nucleotide sequences were submitted to the GenBank database. The accession numbers of partial core and NS5B sequences were HQ229038 and HQ229604, respectively. HCV genotypes in this study were classified according to the reference strains as follows: 1a: NC004102, EF407419,AB301742, AF511950, EU234064, DQ155558, EU256041, EU781772, D10749; 1b: HM041987, HM041997, EF032894, AB016785, EU155228, U16362, D11355, FJ217354, EF032894, AF145454, EU155305, AB429050, AY587016, D90208, AF176573; 1c: AY051292; 2a: AB047639; 2b: D10988; 3a: D17763, AF046866, X76918, AF525902, AB472164, D14308, D10079, EF543248, AM423015, EF543249, X76918, AY003973, AF506583, D10078, AB327107, AB444431, AB444489; 3b: D37840, HM042021, D37854, D49374, D37853; 3k: D63821; 4a: NC009825, DQ418788; 4d: DQ418786; 5a: Y13184, NC009826; 6a: DQ480513, AY859526; 6b: D84262; 6c: EF424629; 6d: D84263; 6e: DQ314805; 6f: DQ835760, DQ835764; 6g: D63822, DQ314806; 6h: D84265; 6i: DQ835770, DQ835762; 6j: DQ835761, DQ835769; 6k: DQ278893, D84264; 6l: EF424628; 6m: DQ835765, DQ835763; 6n: AY878652, DQ835768; 6o: EF424627; 6p: EF424626; 6q: EF424625; 6r: EU408328; 6s: EU408329; 6t: EF632071; 6u: EU246940, and 6w: DQ278892.

Results

Clinical Data and Patient Background

A total of 356 serum samples collected from patients with detectable HCV-RNA were subjected to RT-PCR amplification and sequencing of the core and NS5B regions. Subject age ranged between 17 and 73 years and the mean age was 43.01 ± 10.9. Out of 187 patients with

Table 1. Genotype of HCV determined in this study

Core genotype	NS5B genotype											
	1a	1b	2a	3a	3b	6e	6f	6i	6j	6n	ND	total
1a	75											75 (21.1)
1b	1	48										49 (13.8)
2a			2									2 (0.6)
3a				130	1						5	136 (38.2)
3b					17						1	18 (5.1)
6e						1						1 (0.3)
6f							37				2	39 (11)
6i								7				7 (2.0)
6j									7			7 (2.0)
6n										17		17(4.8)
ND		1		2	2							5 (1.4)
Total, n (%)	76 (21.3)	49 (13.3)	2 (0.6)	132 (37.1)	20 (5.6)	1 (0.3)	37 (10.4)	7 (2.0)	7 (2.0)	17 (4.8)	8 (2.2)	356 (100)

Values are given as n (%). Genotypes are classified based on core and NS5B sequences. ND = not detected.

clinical reports, 92% were diagnosed with chronic liver disease, 5.3% with cirrhosis and 2.7% with HCC. The mean age of the patients in the HCC group was higher than in the chronic hepatitis and cirrhosis group (57, 43.7 and 46.3 years, respectively). Among patients in the chronic hepatitis group, males were predominant with a 2:1 male-to-female ratio. Approximately 92% of the patients lived in the central area of Thailand. One hundred and thirty (36.5%) subjects had known risk factors such as blood transfusion (33.1%), tattooing (27.7%), intravenous drug use (26.9%), unsafe medical injection (23.8%), shaving by a barber (23.1%), prostitution (13%), needle stick injury (11%), HIV coinfection (1.5%) and a spouse with HIV/HCV (1.5%) (see online supplementary tables 1, 2; for all online suppl. material, see www.karger.com/doi/10.1159/000351621). Among all risk factors, intravenous drug use incurred the greatest diversity of HCV genotypes identified (genotypes 1, 2, 3 and 6). In cases where the patient's background showed that more than one risk factor could be associated with multiple routes of transmission, the exact route could not be specified in this study (suppl. table 3).

HCV Genotype

Phylogenetic analysis based on partial core and NS5B sequences (n = 356 sequences) showed that most Thai patients carried subtype 3a (n = 137, 38.5%), followed by subtype 1a (n = 75, 21.1%), 1b (n = 49, 13.8%), genotype 6 (n = 71, 19.9%) and subtype 3b (n = 20, 5.6%). Genotype 6 was classified as 6e (n = 1, 0.3%), 6f (n = 39, 11%), 6i (n = 7, 2.0 %), 6j (n = 7, 2.0%) and 6n (n = 17, 4.8%) (suppl. fig. 1). Genotype 2 was detected in only 2 cases. Two samples, CUTH45 and CUTH233, showed discordant subtypes based on the core and NS5B, in that one sample was 1b/1a and the other was 3a/3b, respectively (table 1). To test that the discordant core and NS5B genotypes were not an artifact of a selected substitution model, the genotype was reclassified by other phylogenetic and nucleotide substitution models. The results showed the same classification was given to both discordant samples by the maximum likelihood trees and the neighbor-joining trees (suppl. fig. 2, 3).

HCV genotype 3 could be detected in all regions of the country; genotype 2 was identified in 2 patients residing in the central area, while genotypes 1 and 6 were not found in the southern and western regions, (suppl. table 2). These results may be due to the fact that most of the patients in this study were recruited from Chulalongkorn Hospital, hence central Thailand. In addition, HCV genotypes 1 and 3 were found in all stages of liver disease, while genotype 2 was found only in chronic hepatitis and genotype 6 was not detected in any HCC patients (suppl. table 1).

Phylogenetic Analysis of HCV Subtype 3a

HCV subtype 3a isolated in this study was analyzed in comparison with reference sequences available from the Los Alamos HCV database. Figure 1 shows the neighbor-

joining tree estimated from the partial core and NS5B sequences. In total, 136 core and 132 NS5B sequences clustered together and were classified as subtype 3a (table 1). In this clade, subbranches exhibited a phylogeographic structure by grouping samples from the same origin together, e.g. HCV strains from the former Soviet Union, Pakistan and Thailand (fig. 1a, b).

The phylogenetic tree of partial core sequences isolated in this study had 92 isolates in the Thai cluster, while the tree constructed from NS5B sequences had 82 isolates (fig. 1a, b). To define these Thai clusters more clearly, 74 samples from both the core and NS5B sequences from these same clusters were selected for further evolutionary analysis.

Evolutionary Analysis of HCV Subtype 3a

The NS5B dataset was analyzed using BEAST under a strict and uncorrelated lognormal relaxed molecular clock with a coalescence bayesian skyline plot model. Model comparison was undertaken through comparison of estimated marginal likelihoods of each model calculated by the Tracer v. 1.5 program. The results show that the relaxed molecular clock had a higher marginal likelihood than the strict model, ln P (model|data) was –3,028.84 and –3,039.35, respectively (log10 Bayes factor of >4.5). Using the data from the relaxed clock model, the origin of the most recent common ancestor of the HCV subtype 3a strains in Thailand existed around 200 years ago (95% credible interval, 573–15 years ago). The mean rate under relaxed clock (ucld.mean) was 9.28×10^{-4} (95% credible region: 1.23×10^{-6} to 2.04×10^{-3}) and the standard deviation (ucld.stdev) was 0.29 (95% credible region: 6.51×10^{-2} to 0.49). If the ucld.stdev is zero, it means no variation in the rate among branches. Rate heterogeneity among the site (α) of NS5B was 0.267 while the coefficient of variation (COV) was 0.3 (95% credible region: 0.05–0.49), which showed heterogeneity among branches. COV measures the rate variation among lineages. A small value of COV represents less rate variation and close to clock-like evolution. So, the evolution will be the strict molecular clock if the COV is equal to zero. Our COV was close to the previous reported value for HCV genotype 6 and Dengue virus [35, 36]. Covariance parameter of the relaxed clock was –0.0079 (95% credible region: –0.1642 to 0.151).

Figure 2 shows the bayesian skyline plot estimated from the NS5B dataset. The effective population size was relatively constant from the early 20th century before changing to exponential growth in the mid-1970s. From the early 1980s to the present the rate has been on a relatively slow decline and seems to be steady after the year 1990.

Discussion

In this study, we have described the genotypic and evolutionary analysis of HCV-infected patients in Thailand. While the severity of liver disease was associated with advanced age, the number of cirrhotic and HCC cases remained small. However, previous studies have found that the mean age of patients with HCC is significantly higher than that of chronic hepatitis patients, suggesting that protracted infection may increase the likelihood of severe complications [13, 15, 30].

In Thailand, HCV genotype 3 was the predominant genotype with subtype 3a being the most common subtype. Genotype 1 and 6 variants were also prevalent, followed by subtype 3b and subtypes 2a and 2c [12, 37]. However, although the genotypic distribution of HCV found in this study is similar to previous reports, these results likely do not accurately represent Thailand's overall genotype distribution because most of the examined samples were obtained from patients living in the central region of Thailand. Also, two samples, 1b/1a and 3a/3b, showed discordance between subtypes based on the core and NS5B regions (table 1, suppl. fig. 1, 2). Intragenotypic recombination of HCV has been reported before, thus additional analysis should be performed to identify the recombination breakpoint of these discrepant samples to confirm the recombination event [38].

Some of the HCV genotypes show phylogeographic distribution, e.g. genotype 2 is common in Africa and genotype 6 is common in East Asia [29, 35]. Phylogenetic analysis indicated that there was a HCV subtype 3a-specific cluster for Thai strains (fig. 1), which may be attributable to a specific route of transmission. In addition, these strains shared a most recent common ancestor that existed approximately 200 years ago. Based on our phylogenetic trees (fig. 1), the Thai subtype 3a strains did not form a monophyletic cluster, thus various 3a strain ancestors may have been introduced into the country in varying regions and at different time points. However, because most of the HCV subtype 3a stains were collected from central Thailand, we were unable to analyze the possible entry point of the original HCV strain into Thailand, while the emergence of subtype 3a in Thailand may be related to the introduction of Western medical practices such as vaccinations and operations [39].

Phylogenetic reconstruction (fig. 1) showed the Thai and Indian/Pakistani clusters in close proximity, which supports the argument of a close relationship between the HCV subtype 3a viruses circulating in those countries. Historically, the Indian culture has had a profound influ-

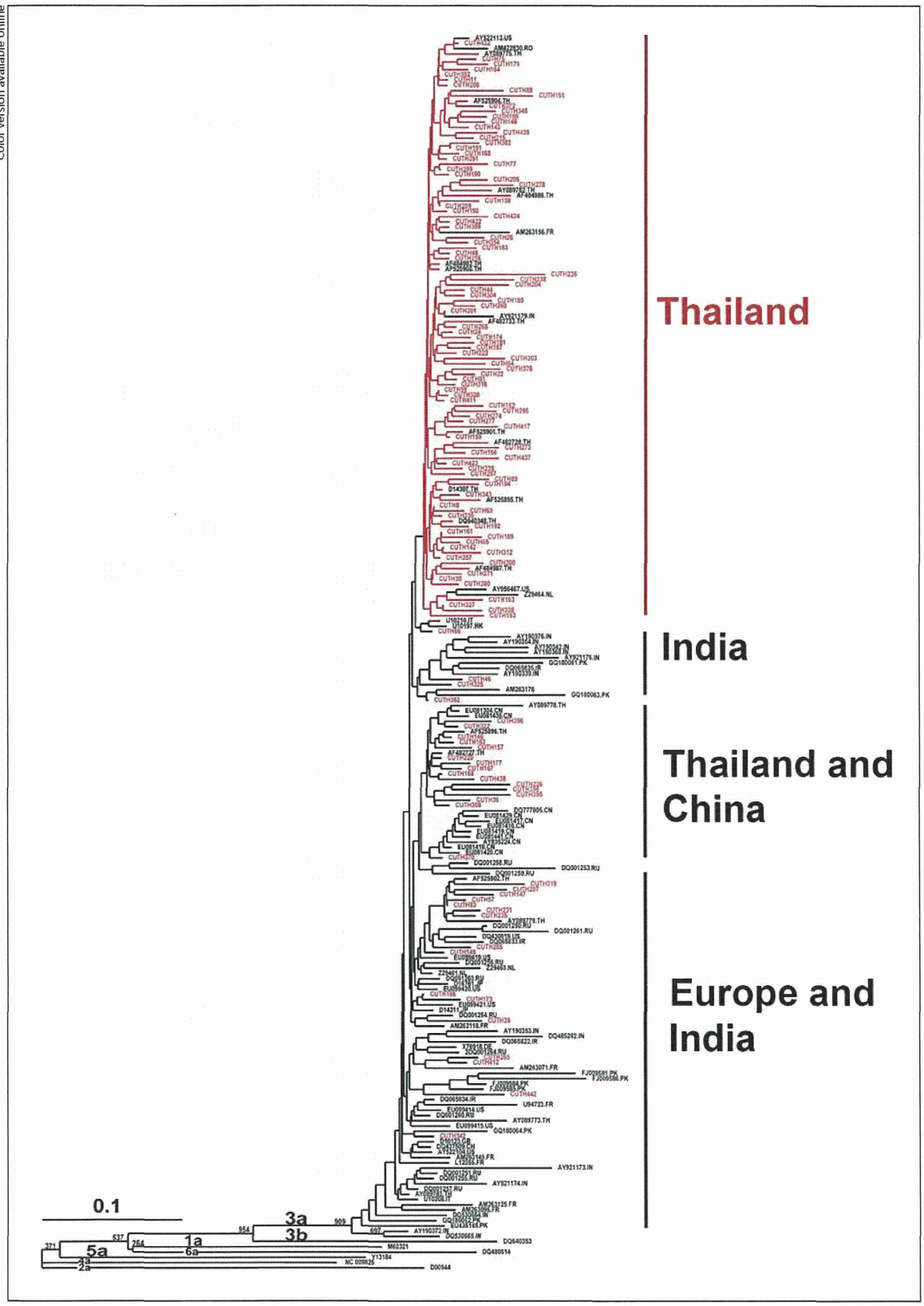
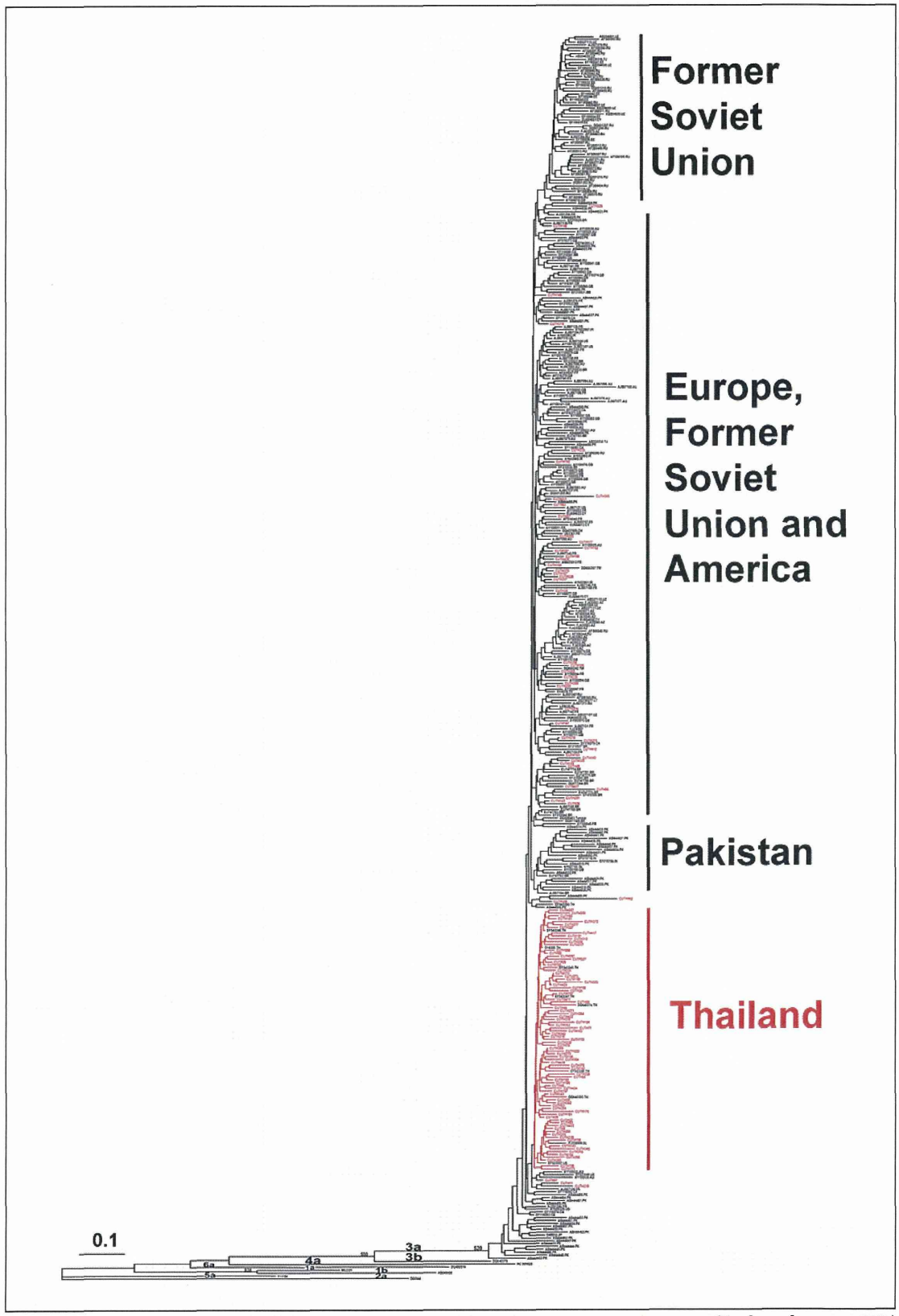


Fig. 1. Phylogenetic tree constructed based on the core (**a**) and NS5B (**b**) region of HCV subtype 3a. Thai isolates identified in this study are labeled in red. The red branches represent a particular cluster of Thai which subjected to evolutionary analysis. Isolates of the same origin tend to cluster together and their countries are shown as abbreviation names. *AU* = Australia; *AZ* = Azerbaijan; *BR* = Brazil; *CA* = Canada; *CH* = Switzerland; *CN* = China; *CY* = Cyprus; *EE* = Estonia; *ES* = Spain; *FR* = France; *GB* = United Kingdom; *IN* = India; *IR* = Iran; *JP* = Japan; *LK* = Sri Lanka; *LT* = Lithuania; *NL* = The Netherlands; *PK* = Pakistan; *RU* = Russia; *TH* = Thailand; *TJ* = Tajikistan; *TN* = Tunisia; *TW* = Taiwan; *US* = United States; *UZ* = Uzbekistan.



Color version available online

(For legend see page 289.)

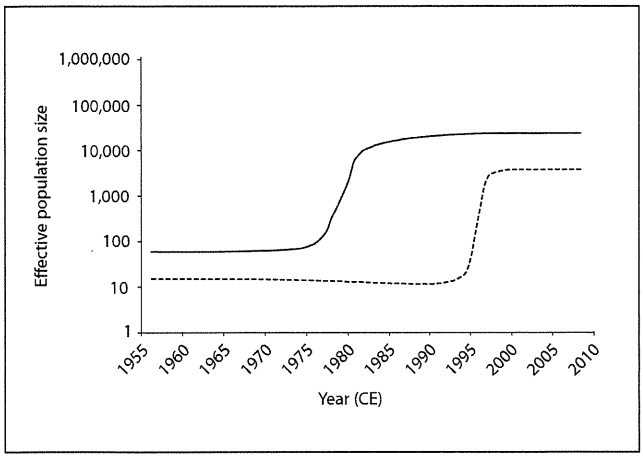


Fig. 2. Bayesian skyline plot for HCV subtype 3a of the Thai cluster. The black line indicates the estimated median of effective numbers of infection based on the NS5B gene and the dotted lines represent 95% highest posterior density confidence interval.

ence on Thai culture, language, ceremony and food. In addition, there has been a history of trade migration between both countries, which may have resulted in a long period of mutual HCV transmission. The widespread introduction of HCV subtype 3a in the Thai population occurred around the mid-1970s and was later introduced to Pakistan [11]. A prior study suggested that the subtype 3a population in Pakistan is older than it is in other countries, and helps contribute to the high number of HCC patients in that country. Khan et al. [11] estimated that the 3a subtype was introduced to the Pakistani population around the 1950s and thus prior to its emergence in the former Soviet Union (ca. 1960s) [30] and Thailand (ca. 1970s). So, it is possible that the most recent common ancestor may have existed in this area.

While 81% of HCV patients in Pakistan carry HCV subtype 3a [11], the prevalence of subtype 3a in India varies upon the sampling population and location. For example, in the northeastern part of India, HCV subtype 3a constitutes around 8% of all genotypes [40]; however, in North and Central India the prevalence of genotype 3 is around 80% [41]. Also, in Bangladesh, an analysis of 168 samples showed that subtype 3b was most prevalent (45.5%), followed by subtype 3a (18.7%) [42]. The prevalence of HCV subtype 3a in the Indian subcontinent is in contrast to reports on Southeast Asian countries – except Thailand – in which the most prevalent genotypes are genotypes 1 and 6 [25, 32, 43–46].

Bayesian phylogeography has provided evidence that HCV subtype 3a was transmitted via heroin trafficking and IDU stemming from the Golden Triangle and Golden Crescent to the Chinese provinces of Yunnan in 1981 and Xinjiang in 1990, which are adjacent to the Golden Triangle and Golden Crescent, respectively. Those two provinces served as a hub for the drug trade and also played an important role in the viral transmission of viruses such as HIV and HCV to other regions of China and Asian [47]. In addition, subtypes 1a and 3a were proposed to have been introduced from an endemic area to the industrial nations by IDU [18]. According to all of these studies, HCV subtype 3a may circulate in an endemic area for a long period and then migrate to other parts of the world through trading, population migration and drug trafficking with subsequent IDU.

As evidenced by our molecular clock estimation, Thai HCV subtype 3a strains have experienced a rapid expansion of their effective population from the mid-1970s to the early 1980s (fig. 2). This time period of expansion is consistent with an influx of IDUs in Thailand as a result of US Army deployment in Southeast Asia during the course of the Vietnam War (1955–1975). At that time, the US Army set up camp at several locations throughout the North and Northeast of Thailand, and the use of stimulants via intravenous administration was widespread [48]. More recently, however, the trend of drug administration has changed from needle to noninjection (non-IDU) practices such as inhaling or oral uptake. Specifically, methamphetamine use has dramatically risen since the 1990s and has largely replaced intravenous drug use. HCV prevalence among IDUs (approx. 86%), however, is still proportionally much higher than non-IDUs (approx. 5%) [21, 49].

Thus, even though we propose that needle sharing may be implicated as the major route of transmission of subtype 3a in Thailand, other factors may have a part in maintaining the present day transmission rates. Risk factor profiles of our patients showed that blood transfusion represented the most profound risk factor in this study (suppl. table 3). Thus, we hypothesize that while intravenous drug use may have been the major cause of the viral expansion in the Thai population during the mid-1970s through the 1980s, contaminated blood products likely play a role in maintaining the current and new viral strains in the population.

In the past, anonymous blood donation was not a popular practice among Thai people, who traditionally preferred to receive blood from their friends or relatives (http://www.blooddonationthai.com/, in Thai). Howev-

er, by 1985 this behavior had changed and blood transfusions from anonymous donors gradually replaced the old tradition. In 1986, donors from convicts were excluded, and in 1990 the National Blood Bank, Thai Red Cross Society implemented universal screening for anti-HCV. At this time, it was found that 2% of first-time donors were positive for HCV antibodies, though this gradually declined to 0.5% by 2009 [50]. Presently, the blood screening policy of the National Blood Center consists of questionnaires to exclude high-risk donors, anti-HCV serology screening of all blood units using chemiluminescent immune assay with some negative samples being further screened by nucleic acid amplification technology [50]. Furthermore, coverage of nucleic acid amplification technology testing throughout the country has been increased from 34% in 2009 to 80% in 2012 (The 21st Academic Annual Meeting of National Blood Centre 2013, Bangkok, Thailand). However, even though the rate of HCV infection among current donors in Thailand is low due to the blood screening policy implemented, the HCV seroprevalence survey was still as high as 2.2% in 2004 [12].

In 2007, the Bureau of Epidemiology reported that HCV was endemic in Thailand's Phetchabun Province and proffered that the route of transmission was due to an unhygienic medical injection procedure [51] given by inadequately trained medical personnel [52]. The infection rate was 70% for intravenous drug users compared to a rate of 0.98% in new blood donors [21]. Thus, initially, needle sharing combined with receiving contaminated blood products significantly escalated the risks of HCV transmission; however, transfusion-related HCV infection has been under control since the implementation of the screening program in 1990. In addition, the first 2 cases of HIV were first reported in Thailand in 1984, and since then health and biosafety education concerning HIV includes information on HCV also [53].

In 2009, the Thailand National Cancer Institute reported that liver and bile duct cancer was the third most common cause of cancer in Thailand, ranking as the third most common cause in males (12.4%) and as the fifth most common cause in females (4%) [54]. The report also showed that most hepatitis cases were the result of infection with HBV (approx. 51.9%), followed by HCV (approx. 11.8%) and, to a lesser extent, HAV, HDV, HEV and others [55]. Therefore, although HCV is generally the major cause of new HCC cases in developed countries [13], HBV is the most predominant cause of HCC in Thailand [56]. Due to the course of disease progression, HCV-related liver disease may take 20–30 years before progressing to liver cirrhosis or HCC [2, 3, 27]. Thus, the spread of HCV in Thailand during the 1970s and 1980s may soon manifest itself phenotypically. Fortunately, however, since 2012 the National Health Security Office (NHSO) has launched a policy of free HCV treatment by registered specialists for chronic hepatitis patients with HCV genotype 2 or 3, which are the genotypes most likely to be cured by the standard viral therapy of pegylated interferon plus ribavirin. Accordingly, if most of the favorable HCV genotype patients can be accessed and receive treatment, the incidence of HCV-related diseases may not be as high as expected in the future.

Similar to previous reports, our study indicates that HCV subtype 3a is the most prevalent strain in Thailand followed by genotypes 1 and 6. Evolutionary analysis based on the coalescence theory estimated that common HCV subtype 3a was introduced and spread into the Thai population during the mid-1970s to the early 1980s. This event coincided with the Vietnam War period which is also when intravenous drug use and needle sharing was widespread. Sharing needles may have been a primary source of the HCV epidemic in the country, and receiving contaminated blood products may have been the primary factor for high HCV prevalence in the Thai population. However, HCV prevalence seems to have steadily declined since anti-HCV antibody screening was implemented in blood donation centers. Our results suggest that the cirrhosis and HCC incidence of this population may increase in the future unless effective treatment and public health policy is introduced to help prevent further disease progression.

Acknowledgements

This research was supported by the National Research Fund; the Center of Excellence in Clinical Virology Fund, Faculty of Medicine, Chulalongkorn University; Thai Red Cross Society; Postdoctoral Ratchadaphiseksomphot Endowment Fund; Research Unit of Hepatitis and Liver Cancer, Chulalongkorn University; Chulalongkorn University Centenary Academic Development Project, Integrated Innovation Academic Center; Chulalongkorn University Centenary Academic Development Project (CU56-HR01); the RGJ program and the Thailand Research Fund (DPG5480002 and BRG5580005), and the National University Research Fund, Office of the Higher Education Commission (HR1155A-55 and HR1162A-55). We would also like to express our gratitude to the hospital in Phetchabun Province for collecting the specimens. We also would like to thank Ms. Petra Hirsch and Mr. Brian Padfield Muchmore for reviewing the manuscript.

References

1 Lavanchy D: The global burden of hepatitis C. Liver Int 2009;29:74–81.
2 Hoofnagle JH: Course and outcome of hepatitis C. Hepatology 2002;36:S21–S29.
3 Seeff LB: Natural history of chronic hepatitis C. Hepatology 2002;36:S35–S46.
4 Trinchet JC, Ganne-Carrie N, Nahon P, N'Kontchou G, Beaugrand M: Hepatocellular carcinoma in patients with hepatitis C virus-related chronic liver disease. World J Gastroenterol 2007;13:2455–2460.
5 Fattovich G, Stroffolini T, Zagni I, Donato F: Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 2004;127:S35–S50.
6 Yoshizawa H: Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 2002;62:8–17.
7 Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, Halfon P, Inchauspe G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin IT, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A: Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 2005; 42:962–973.
8 Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, Beall E, Yap PL, Kolberg J, Urdea MS: Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol 1993;74:2391–2399.
9 Pybus OG, Drummond AJ, Nakano T, Robertson BH, Rambaut A: The epidemiology and iatrogenic transmission of hepatitis C virus in Egypt: a bayesian coalescent approach. Mol Biol Evol 2003;20:381–387.
10 Simmonds P: Genetic diversity and evolution of hepatitis C virus – 15 years on. J Gen Virol 2004;85:3173–3188.
11 Khan A, Tanaka Y, Azam Z, Abbas Z, Kurbanov F, Saleem U, Hamid S, Jafri W, Mizokami M: Epidemic spread of hepatitis C virus genotype 3a and relation to high incidence of hepatocellular carcinoma in Pakistan. J Med Virol 2009;81:1189–1197.
12 Sunanchaikarn S, Theamboonlers A, Chongsrisawat V, Yoocharoen P, Tharmaphornpilas P, Warinsathien P, Sinlaparatsamee S, Paupunwatana S, Chaiear K, Khwanjaipanich S, Poovorawan Y: Seroepidemiology and genotypes of hepatitis C virus in Thailand. Asian Pac J Aller Immunol 2007;25:175–182.
13 Zein NN: Clinical significance of hepatitis C virus genotypes. Clin Microbiol Rev 2000;13: 223–235.
14 Akkarathamrongsin S, Praianantathavorn K, Hacharoen N, Theamboonlers A, Tangkijvanich P, Tanaka Y, Mizokami M, Poovorawan Y: Geographic distribution of hepatitis C virus genotype 6 subtypes in Thailand. J Med Virol 2010;82:257–262.
15 Elkady A, Tanaka Y, Kurbanov F, Sugauchi F, Sugiyama M, Khan A, Sayed D, Moustafa G, Abdel-Hameed AR, Mizokami M: Genetic variability of hepatitis C virus in South Egypt and its possible clinical implication. J Med Virol 2009;81:1015–1023.
16 Lu L, Nakano T, Li C, Fu Y, Miller S, Kuiken C, Robertson BH, Hagedorn CH: Hepatitis C virus complete genome sequences identified from China representing subtypes 6k and 6n and a novel, as yet unassigned subtype within genotype 6. J Gen Virol 2006;87:629–634.
17 Romano CM, de Carvalho-Mello IM, Jamal LF, de Melo FL, Iamarino A, Motoki M, Pinho JR, Holmes EC, de Andrade Zanotto PM: Social networks shape the transmission dynamics of hepatitis C virus. PLoS One 2010;5: e11170.
18 Pybus OG, Cochrane A, Holmes EC, Simmonds P: The hepatitis C virus epidemic among injecting drug users. Infect Genet Evol 2005;5:131–139.
19 Kurbanov F, Tanaka Y, Avazova D, Khan A, Sugauchi F, Kan N, Kurbanova-Khudayberganova D, Khikmatullaeva A, Musabaev E, Mizokami M: Detection of hepatitis C virus natural recombinant RF1_2k/1b strain among intravenous drug users in Uzbekistan. Hepatol Res 2008;38:457–464.
20 Shustov AV, Kochneva GV, Sivolobova GF, Grazhdantseva AA, Gavrilova IV, Akinfeeva LA, Rakova IG, Aleshina MV, Bukin VN, Orlovsky VG, Bespalov VS, Robertson BH, Netesov SV: Molecular epidemiology of the hepatitis C virus in western Siberia. J Med Virol 2005;77:382–389.
21 Verachai V, Phutiprawan T, Theamboonlers A, Chinchai T, Tanprasert S, Haagmans BL, Osterhaus AD, Poovorawan Y: Prevalence and genotypes of hepatitis C virus infection among drug addicts and blood donors in Thailand. Southeast Asian J Trop Med Public Health 2002;33:849–851.
22 Rosenberg NA, Nordborg M: Genealogical trees, coalescent theory and the analysis of genetic polymorphisms. Nat Rev Genet 2002;3: 380–390.
23 Pybus OG, Charleston MA, Gupta S, Rambaut A, Holmes EC, Harvey PH: The epidemic behavior of the hepatitis C virus. Science 2001;292:2323–2325.
24 Jutavijittum P, Jiviriyawat Y, Yousukh A, Pantip C, Maneekarn N, Toriyama K: Genotypic distribution of hepatitis C virus in voluntary blood donors of northern Thailand. Southeast Asian J Trop Med Public Health 2009;40:471–479.
25 Lwin AA, Shinji T, Khin M, Win N, Obika M, Okada S, Koide N: Hepatitis C virus genotype distribution in Myanmar: predominance of genotype 6 and existence of new genotype 6 subtype. Hepatol Res 2007;37:337–345.
26 Hubschen JM, Jutavijittum P, Thammavong T, Samountry B, Yousukh A, Toriyama K, Sausy A, Muller CP: High genetic diversity including potential new subtypes of hepatitis C virus genotype 6 in Lao People's Democratic Republic. Clin Microbiol Infect 2011;17:E30–E34.
27 Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T, Alter HJ: Inaugural article: a comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA 2002;99:15584–15589.
28 Nakano T, Lu L, He Y, Fu Y, Robertson BH, Pybus OG: Population genetic history of hepatitis C virus 1b infection in China. J Gen Virol 2006;87:73–82.
29 Markov PV, Pepin J, Frost E, Deslandes S, Labbe AC, Pybus OG: Phylogeography and molecular epidemiology of hepatitis C virus genotype 2 in Africa. J Gen Virol 2009;90: 2086–2096.
30 Tanaka Y, Kurbanov F, Mano S, Orito E, Vargas V, Esteban JI, Yuen MF, Lai CL, Kramvis A, Kew MC, Smuts HE, Netesov SV, Alter HJ, Mizokami M: Molecular tracing of the global hepatitis C virus epidemic predicts regional patterns of hepatocellular carcinoma mortality. Gastroenterology 2006;130: 703–714.
31 Theamboonlers A, Chinchai T, Bedi K, Jantarasamee P, Sripontong M, Poovorawan Y: Molecular characterization of hepatitis C virus (HCV) core region in HCV-infected Thai blood donors. Acta Virol 2002;46:169–173.
32 Akkarathamrongsin S, Praianantathavorn K, Hacharoen N, Theamboonlers A, Tangkijvanich P, Poovorawan Y: Seroprevalence and genotype of hepatitis C virus among immigrant workers from Cambodia and Myanmar in Thailand. Intervirology 2011;54:10–16.
33 Kuiken C, Yusim K, Boykin L, Richardson R: The Los Alamos hepatitis C sequence database. Bioinformatics 2005;21:379–384.
34 Mizokami M, Tanaka Y, Miyakawa Y: Spread times of hepatitis C virus estimated by the molecular clock differ among Japan, the United States and Egypt in reflection of their distinct socioeconomic backgrounds. Intervirology 2006;49:28–36.
35 Pybus OG, Barnes E, Taggart R, Lemey P, Markov PV, Rasachak B, Syhavong B, Phetsouvanah R, Sheridan I, Humphreys IS, Lu L, Newton PN, Klenerman P: Genetic history of hepatitis C virus in East Asia. J Virol 2009;83: 1071–1082.
36 Drummond AJ, Ho SY, Phillips MJ, Rambaut A: Relaxed phylogenetics and dating with confidence. PLoS Biol 2006;4:e88.
37 Kanistanon D, Neelamek M, Dharakul T, Songsivilai S: Genotypic distribution of hepatitis C virus in different regions of Thailand. J Clin Microbiol 1997;35:1772–1776.
38 Cristina J, Colina R: Evidence of structural genomic region recombination in hepatitis C virus. Virol J 2006;3:53.

39 Charuluxananan SC, Chentanezb V: History and evolution of Western medicine in Thailand. Asian Biomed 2007;1:5.
40 Medhi S, Goswami B, Das AK, Singh TB, Husain SA, Sehgal A, Kar P: New insights into hepatitis C virus infection in the tribal-dominant part of northeast India. Arch Virol 2012; 157:2083–2093.
41 Hissar SS, Goyal A, Kumar M, Pandey C, Suneetha PV, Sood A, Midha V, Sakhuja P, Malhotra V, Sarin SK: Hepatitis C virus genotype 3 predominates in North and Central India and is associated with significant histopathologic liver disease. J Med Virol 2006;78: 452–458.
42 Islam JH, Jamiruddin A, Ahmed R, et al.: Prevalence of hepatitis C virus genotypes in Bangladesh. 13th Annual Scientific Conference (ASCON), Dhaka, 2011, SP132, p 197.
43 Pham DA, Leuangwutiwong P, Jittmittraphap A, Luplertlop N, Bach HK, Akkarathamrongsin S, Theamboonlers A, Poovorawan Y: High prevalence of hepatitis C virus genotype 6 in Vietnam. Asian Pac J Allergy Immunol 2009;27:153–160.
44 Pybus OG, Rambaut A: Evolutionary analysis of the dynamics of viral infectious disease. Nat Rev Genet 2009;10:540–550.
45 Utama A, Tania NP, Dhenni R, Gani RA, Hasan I, Sanityoso A, Lelosutan SA, Martamala R, Lesmana LA, Sulaiman A, Tai S: Genotype diversity of hepatitis C virus (HCV) in HCV-associated liver disease patients in Indonesia. Liver Int 2010;30:1152–1160.
46 Kageyama S, Agdamag DM, Alesna ET, Abellanosa-Tac-An IP, Corpuz AC, Telan EF, Que ER, Leano PS, Jereza LD, Emphasis YE, Prasetyo AA, Tanimoto T, Ichimura H: Tracking the entry routes of hepatitis C virus as a surrogate of HIV in an HIV-low prevalence country, the Philippines. J Med Virol 2009;81: 1157–1162.
47 Liu J, Zhang C: Phylogeographic analyses reveal a crucial role of Xinjiang in HIV-1 CRF07_BC and HCV 3a transmissions in Asia. PLoS One 2011;6:e23347.
48 Pattha P: The Impact of American Bases on Socio-Economic Condition of Udornthani, A.D. 1962–1977; thesis, Silpakorn University, Bangkok, 2007, pp 48, 61.
49 Jittiwutikarn J, Thongsawat S, Suriyanon V, Maneekarn N, Celentano D, Razak MH, Srirak N, Vongchak T, Kawichai S, Thomas D, Sripaipan T, Netski D, Ananthakrishnan A, Nelson KE: Hepatitis C infection among drug users in northern Thailand. Am J Trop Med Hyg 2006;74:1111–1116.
50 Chimparlee N, Oota S, Phikulsod S, Tangkijvanich P, Poovorawan Y: Hepatitis B and hepatitis C virus in Thai blood donors. Southeast Asian J Trop Med Public Health 2011;42: 609–615.
51 Buathong R: Hepatitis. Annu Epidemiol Surveill Rep 2007:88–92.
52 Tanwandee T, Piratvisuth T, Phornphutkul K, Mairiang P, Permpikul P, Poovorawan Y: Risk factors of hepatitis C virus infection in blood donors in Thailand: a multicenter case-control study. J Med Assoc Thai 2006; 89:S79–S83.
53 Limsuwan A, Kanapa S, Siristonapun Y: Acquired immune deficiency syndrome in Thailand. A report of two cases. J Med Assoc Thai 1986;69:164–169.
54 Attasara PB, et al: Hospital-Based Cancer Registry 2008. Bangkok, National Cancer Institute Thailand, 2009.
55 Santayakorn S: Hepatitis. Annu Epidemiol Surveill Rep 2009;100–104.
56 Tangkijvanich P, Mahachai V, Suwangool P, Poovorawan Y: Gender difference in clinicopathologic features and survival of patients with hepatocellular carcinoma. World J Gastroenterol 2004;10:1547–1550.

OPEN ACCESS

Open Access
Scan to access more free content

ORIGINAL ARTICLE

Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

Tsunamasa Watanabe,[1] Fuminaka Sugauchi,[2] Yasuhito Tanaka,[1] Kentaro Matsuura,[3] Hiroshi Yatsuhashi,[4] Shuko Murakami,[1] Sayuki Iijima,[1] Etsuko Iio,[3] Masaya Sugiyama,[5] Takashi Shimada,[6] Masakazu Kakuni,[6] Michinori Kohara,[7] Masashi Mizokami[5]

▶ Additional supplementary files are published online only. To view these files please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2012-302553).

[1]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[2]Department of Gastroenterology, Nagoya City Koseiin Medical Welfare Center, Nagoya, Japan
[3]Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[4]Department of Therapeutic Research, National Hospital Organization (NHO) Nagasaki Medical Center, Nagasaki, Japan
[5]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan
[6]PhoenixBio Co. Ltd., Higashi-Hiroshima, Japan
[7]Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Correspondence to
Dr Masashi Mizokami, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine 1–7–1, Kohnodai, Ichikawa 272–8516, Japan; mmizokami@hospk.ncgm.go.jp

Revised 4 October 2012
Accepted 9 October 2012
Published Online First 7 November 2012

Linked

▶ http://dx.doi.org/10.1136/gutjnl-2012-303904

To cite: Watanabe T, Sugauchi F, Tanaka Y, *et al*. *Gut* 2013;**62**:1340–1346.

ABSTRACT

Objective Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon α (peg-IFN-α) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

Design Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN-α plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN-α for 2 weeks.

Results There were significant differences in the reduction of HCV-RNA levels after peg-IFN-α plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN-α administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

Conclusions As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

Significance of this study

What is already known on this subject?
- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN-α-based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

What are the new findings?
- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN-α was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN-α treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

How might it impact on clinical practice in the foreseeable future?
- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.[1]

The standard therapy for hepatitis C still consists of pegylated interferon-α (peg-IFN-α), administered once weekly, plus daily oral ribavirin for 24–48 weeks in countries where protease inhibitors are not available.[2] This combination therapy is quite successful in patients with HCV genotype 2 or 3

infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.[3 4]

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.[5] Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)-λ3, are associated with a chronic HCV treatment response.[6–10] Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.[11–12]

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals;[13] HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.[14] However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)[15–17] and are suitable for experiments with hepatitis viruses in vivo.[18 19] We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.[20 21]

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN-α treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN-α without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN-α treatment.

MATERIALS AND METHODS

Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City University were enrolled in this study (table 1). Patients received peg-IFN-α2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily), which is the recommended dosage in Japan. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

Table 1 Characteristics of 54 patients infected HCV genotype 1

	IL28B SNP rs8099917		
	TT (n=34)	TG (n=19) + GG (n=1)	p Value
Age (years)	55.6±10.1	54.7±11.3	0.746
Gender (male %)	70	50	0.199
Body mass index (kg/m^2)	24.6±3.1	24.7±3.3	0.870
Viral load at therapy (log IU/ml)	6.0±0.7	5.8±0.8	0.357
SVR rate (%)	50	11	0.012
Serum ALT level (IU/l)	100.3±80.8	79.3±45.0	0.226
Platelet count (×10^4/μl)	17.1±9.0	16.5±5.8	0.771
Fibrosis (F3+4 %)	42	40	0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plains, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,[6–8] was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with 5.0–7.5×10^5 viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).[17] Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.[22] Three different serum samples were obtained from three chronic HCV patients (genotype 1b).[21 22] Each mouse was intravenously infected with serum sample containing 10^5 copies of HCV genotype 1b. Administration of peg-IFN-α2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30 μg/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.[21]

Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis was performed using 2.0 μg of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ($2^{-(\text{delta Ct})}$) was used for quantitation of relative mRNA levels and fold induction.[23 24]

Table 2 Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes.
SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the χ^2 test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann–Whitney U test. Differences were considered significant if p values were less than 0.05.

RESULTS

Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%, p=0.012). The initial HCV serum load was comparable between genotypes TT and TG/GG (6.0±0.7 vs 5.8±0.8 log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age (55.6±10.1 vs 54.7±11.3 years), serum alanine aminotransferase level (100.3±80.8 vs 79.3±45.0 IU/L), platelet count (17.1±9.0 vs 16.5±5.8×10^4/μl) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

Changes in serum HCV-RNA levels in patients treated by peg-IFN-α plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG (−1.08 vs −0.39 log IU/ml, p<0.001). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN-α plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows: −1.58 vs −0.62, p<0.001; −2.35 vs −0.91, p<0.001; −3.48 vs −1.56, p<0.001; −4.53 vs −2.37, p<0.01; −4.93 vs −2.86, p<0.001. Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day 0.94±0.83 vs 0.38±0.40 log IU/ml, p<0.001; Ph2/week 0.08±0.06 vs 0.04±0.03 log IU/ml, p<0.001) (figure 3).

Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN-α

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN-α, we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than 10^6 copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN-α administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected[22] chimeric mice sera was observed between favourable (n=7) and unfavourable

Table 3 Dosage and time schedule of pegIFN-α2a* treatment for HCV genotype 1b infected chimeric mice

				Dose			
Donor hepatocytes†	No of chimeric mice	Inoculum	Test compound	Level (μg/kg)	Concentrtion (μg/ml)	Volume (ml/kg)	Frequency
A	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10

*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.
HCV, hepatitis C virus; peg-IFN-α, pegylated interferon α.

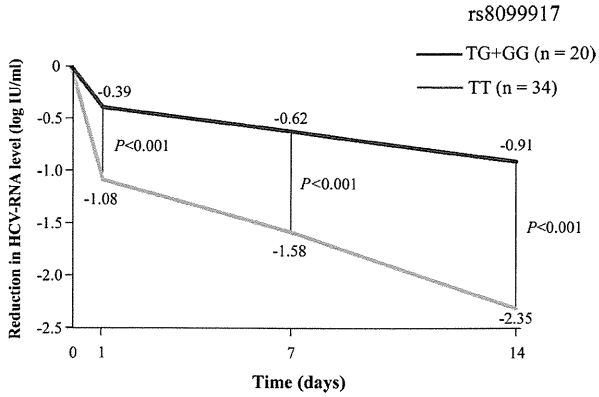


Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

(n=6) *IL28B* genotypes on days 1, 3, 7 and 14 (−1.2 vs −1.3, −1.4 vs −1.4, −1.8 vs −1.7, and −2.3 vs −1.9 log copies/ml) (figure 4A). Moreover, we prepared two additional serum samples from the other HCV-1b patients (serum B and C)[21] to confirm the influence of *IL28B* genotype in early viral kinetics during IFN treatment. After establishing persistent infection with new HCV-1b strains in all chimeric mice, they were also administered four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (figure 4B,C). In a similar fashion, no significant difference in HCV-RNA reduction in chimeric mice sera was observed between favourable and unfavourable *IL28B* genotypes.

Expression levels of ISG in chimeric mice livers

Because chimeric mice have the characteristic of severe combined immunodeficiency, the viral kinetics in chimeric mice sera during IFN treatment could be contributed by the innate immune response of HCV-infected human hepatocytes. Therefore, ISG expression levels in mice livers transplanted with human hepatocytes were compared between favourable and unfavourable *IL28B* genotypes (figure 5).

As shown in figure 5A, ISG expression levels in mice livers were measured at 8 h and 24 h after IFN treatment. The levels

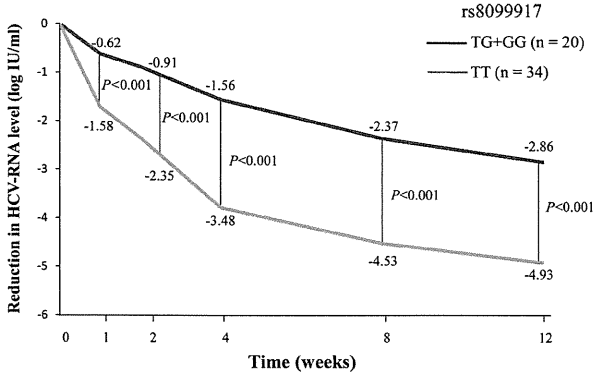


Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

of representative antiviral ISG (eg, myxovirus resistance protein A, oligoadenylate synthetase 1, RNA-dependent protein kinase) and other ISG for promoting antiviral signalling (eg, Toll-like receptor 3, retinoic acid-inducible gene 1) were significantly induced at least 8 h after treatment, and prolonged at 24 h. No significant difference in ISG expression levels in HCV-infected livers was observed between favourable and unfavourable *IL28B* genotypes. The other inoculum for persistent infection of HCV-1b also demonstrated no significant difference in ISG expression levels between favourable and unfavourable *IL28B* genotypes (figure 5B,C). Interestingly, IFN-λ expression levels by treatment of peg-IFN-α were significantly induced in HCV-infected human hepatocytes harbouring the favourable *IL28B* genotype (figure 5 A–C).

DISCUSSION

Several recent studies have demonstrated a marked association between the chronic hepatitis C treatment response[6–9] and SNP (rs8099917, rs8103142 and rs12979860) near or within the region of the *IL28B* gene, which affected the viral dynamics during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals.[13]

It has been reported that when patients with chronic hepatitis C are treated by IFN-α or peg-IFN-α plus ribavirin, HCV-RNA generally declines after a 7–10 h delay.[25] The typical decline is biphasic and consists of a rapid first phase lasting for approximately 1–2 days during which HCV-RNA may fall 1–2 logs in patients infected with genotype 1, and subsequently a slower second phase of HCV-RNA decline.[26] The viral kinetics had a predictive value in evaluating antiviral efficacy.[14] In this study, biphasic decline of the HCV-RNA level during peg-IFN-α treatment was observed in both patients and chimeric mice infected with HCV genotype 1; however, in the first and second phases of viral kinetics, a difference between *IL28B* genotypes was observed only in HCV-infected patients; a more rapid decline in serum HCV-RNA levels after administering peg-IFN-α plus ribavirin was confirmed in patients with the TT genotype of rs8099917 compared to those with the TG/GG genotype.

On the other hand, in-vivo data using the chimeric mouse model showed no significant difference in the reduction of HCV-RNA titers in mouse serum among four different lots of human hepatocytes containing *IL28B* favourable (rs8099917 TT) or unfavourable (rs8099917 TG) genotypes, which was confirmed by the inoculation of two additional HCV strains. These results indicated that variants of the *IL28B* gene in donor hepatocytes had no influence on the response to peg-IFN-α under immunosuppressive conditions, suggesting that the immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

Two recent studies indeed revealed an association between the *IL28B* genotype and the expression level of hepatic ISG in human studies.[27 28] Quiescent hepatic ISG before treatment among patients with the *IL28B* favourable genotype have been associated with sensitivity to exogenous IFN treatment and viral eradication; however, it is difficult to establish whether the hepatic ISG expression level contributes to viral clearance independently or appears as a direct consequence of the *IL28B* genotype. Another recent study addressed this question and the results suggested that there is no absolute correlation with the *IL28B* genotype and hepatic expression of ISG.[29] Our results on the hepatic ISG expression level in immunodeficient chimeric mice also suggested that no significant difference in ISG expression levels was observed between favourable and unfavourable

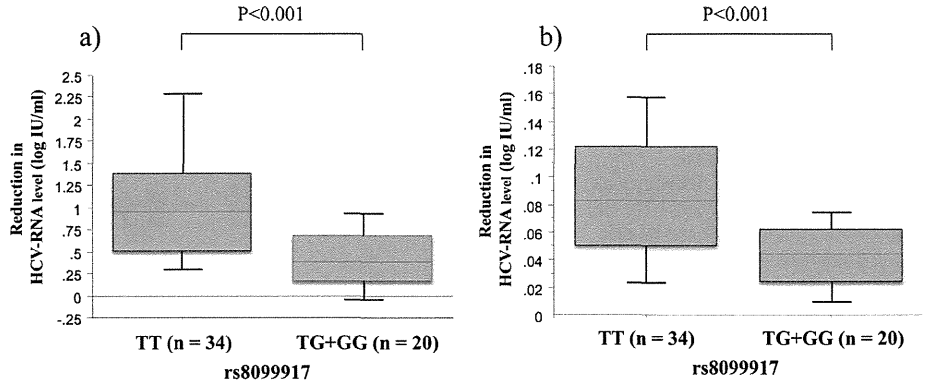


Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

IL28B genotypes. However, these results were not consistent with a previous report using chimeric mice that the favourable *IL28B* genotype was associated with an early reduction in HCV-RNA by ISG induction.[30] The reasons for the discrepancy might depend on the dose and type of IFN treatment, as well as the time point when ISG expression was examined in the liver. In addition, although IFN-λ transcript levels measured in peripheral blood mononuclear cells or liver revealed inconsistent results in the context of an association with the *IL28B* genotype,[7 8] our preliminary assay on the IL28A, IL28B and IL29 transcripts in the liver first indicated that the induction of IFN-λ on peg-IFN-α administration could be associated with the *IL28B* genotype. Therefore, the induction of IFN-λ followed by immune response might contribute to different viral kinetics and treatment outcomes in HCV-infected patients, because no difference was found in chimeric mice without immune response.

It has also been reported that the mechanism of the association of genetic variations in the *IL28B* gene and spontaneous clearance of HCV may be related to the host innate immune response.[11] Interestingly, participants with seroconversion illness with jaundice were more frequently rs8099917 homozygous favourable allele (TT) than other genotypes (32% vs 5%, p=0.047). This suggests that a stronger immune response during the acute phase of HCV infection among patients with the *IL28B* favourable genotype would induce more frequent spontaneous clearance of HCV.

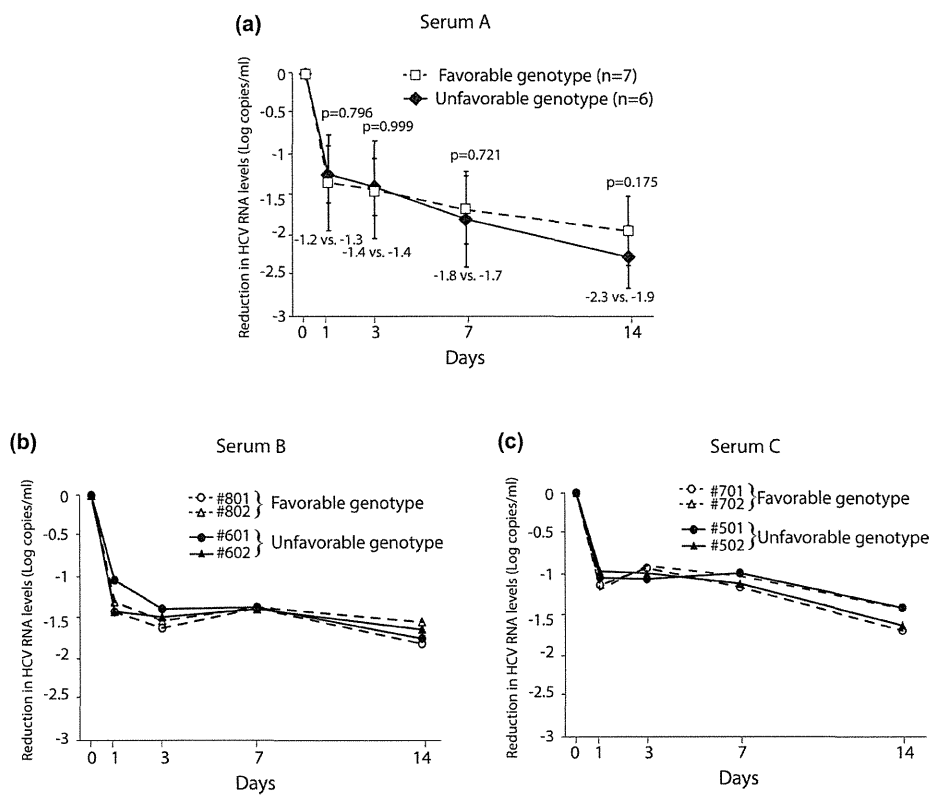


Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.

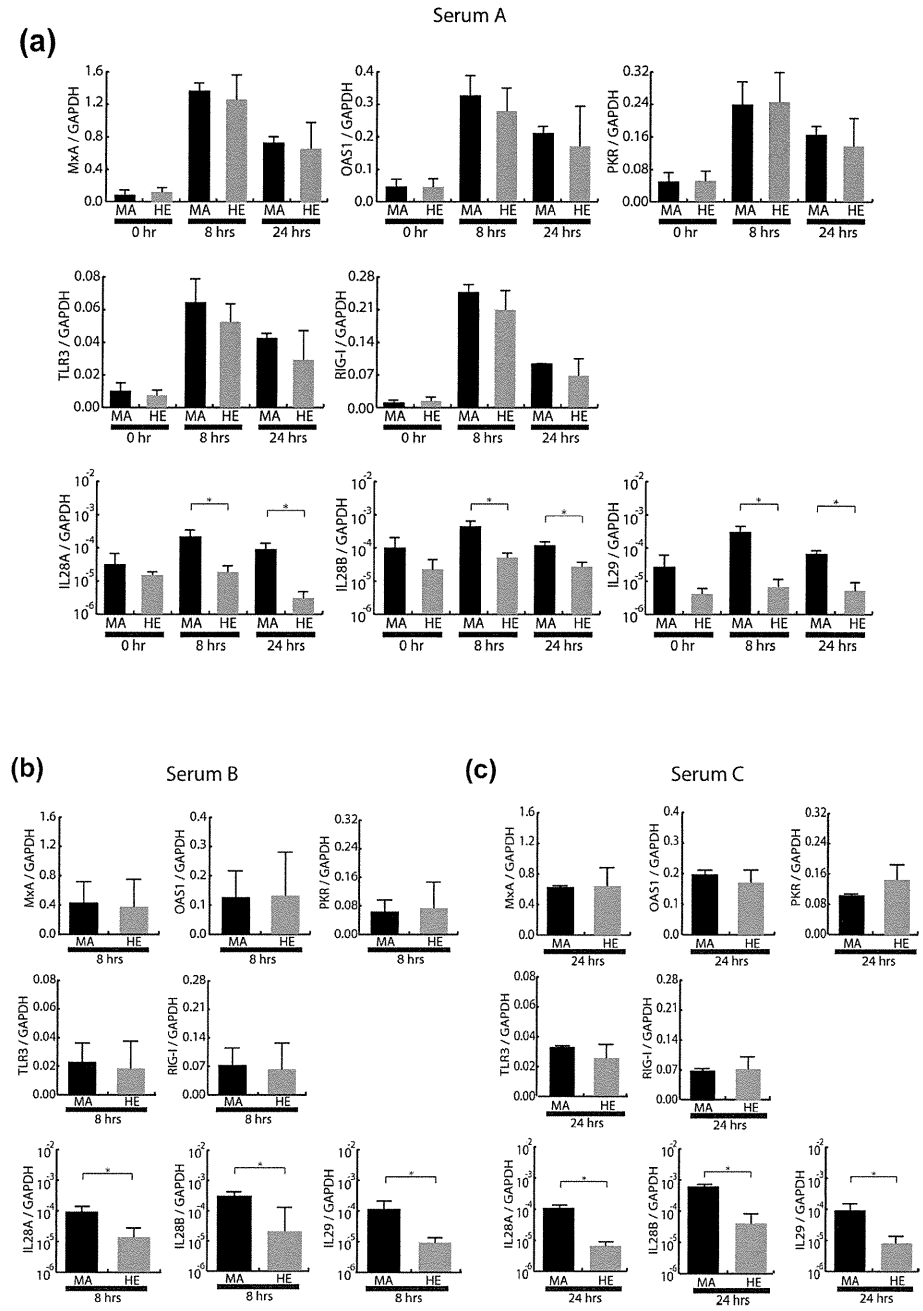


Figure 5 Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon α (peg-IFN-α)-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. Data are represented as mean +SD. (A) Time kinetics of ISG after administration of the peg-IFN-α in serum A-infected chimeric mice (n=3, each genotype). Comparison of ISG expression levels at (B) 8 h in serum B-infected mice and (C) 24 h in serum C-infected mice after administering peg-IFN-α (n=3, each genotype). Predesigned real-time PCR assay of IL28B transcript purchased from Applied Biosystems can be cross-reactive to IL28A transcript. *p<0.05. MxA, myxovirus resistance protein A; OAS1, oligoadenylate synthetase 1; PKR, RNA-dependent protein kinase; RIG-1, retinoic acid-inducible gene 1; TLR3, Toll-like receptor 3.

Taking into account both the above results in acute HCV infection and our results conducted on chimeric mice that have the characteristic of immunodeficiency, it is suggested that the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic hepatitis C patients would be composed of the intact immune system.

Acknowledgements The authors would like to thank Kyoko Ito of Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan for doing the quantification of gene-expression assays.

Contributors YT and MM conceived the study. TW and FS and YT conducted the study equally. TW and FS coordinated the analysis and manuscript preparation. All the authors had input into the study design, patient recruitment and management or mouse management and critical revision of the manuscript for intellectual content. TW, FS and YT contributed equally.

Funding This study was supported by a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan (H22-kannen-005) and the Ministry of Education, Culture, Sports, Science and Technology, Japan, and grant-in-aid for research in Nagoya City University.

Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of each ethics committee at the Nagoya City University and Nagasaki Medical Center (see supplementary information, available online only).

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Ray Kim W. Global epidemiology and burden of hepatitis C. *Microbes Infect* 2002;4:1219–25.

2 Foster GR. Past, present, and future hepatitis C treatments. *Semin Liver Dis* 2004;24(Suppl. 2):97–104.
3 Fried MW, Shiffman ML, Reddy KR, *et al*. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;347:975–82.
4 Manns MP, McHutchison JG, Gordon SC, *et al*. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;358:958–65.
5 Mihm U, Herrmann E, Sarrazin C, *et al*. Review article: predicting response in hepatitis C virus therapy. *Aliment Pharmacol Ther* 2006;23:1043–54.
6 Ge D, Fellay J, Thompson AJ, *et al*. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009;461:399–401.
7 Suppiah V, Moldovan M, Ahlenstiel G, *et al*. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009;41:1100–4.
8 Tanaka Y, Nishida N, Sugiyama M,, *et al* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009;41:1105–9.
9 Rauch A, Kutalik Z, Descombes P, *et al*. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* 2010;138:1338–45.
10 Tanaka Y, Nishida N, Sugiyama M, *et al*. lambda-Interferons and the single nucleotide polymorphisms: a milestone to tailor-made therapy for chronic hepatitis C. *Hepatol Res* 2010;40:449–60.
11 Thomas DL, Thio CL, Martin MP, *et al*. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009;461:798–801.
12 Grebely J, Petoumenos K, Hellard M, *et al*. Potential role for interleukin-28B genotype in treatment decision-making in recent hepatitis C virus infection. *Hepatology* 2010;52:1216–24.
13 Thompson AJ, Muir AJ, Sulkowski MS, *et al*. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. *Gastroenterology* 2010;139:120–29.
14 Layden-Almer JE, Layden TJ. Viral kinetics in hepatitis C virus: special patient populations. *Semin Liver Dis* 2003;23(Suppl. 1):29–33.
15 Heckel JL, Sandgren EP, Degen JL, *et al*. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. *Cell* 1990;62:447–56.
16 Rhim JA, Sandgren EP, Degen JL, *et al*. Replacement of diseased mouse liver by hepatic cell transplantation. *Science* 1994;263:1149–52.
17 Tateno C, Yoshizane Y, Saito N, *et al*. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004;165:901–12.
18 Mercer DF, Schiller DE, Elliott JF, *et al*. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001;7:927–33.
19 Tsuge M, Hiraga N, Takaishi H, *et al*. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 2005;42:1046–54.
20 Kurbanov F, Tanaka Y, Chub E, *et al*. Molecular epidemiology and interferon susceptibility of the natural recombinant hepatitis C virus strain RF1_2k/1b. *J Infect Dis* 2008;198:1448–56.
21 Kurbanov F, Tanaka Y, Matsuura K, *et al*. Positive selection of core 70Q variant genotype 1b hepatitis C virus strains induced by pegylated interferon and ribavirin. *J Infect Dis* 2010;201:1663–71.
22 Inoue K, Umehara T, Ruegg UT, *et al*. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. *Hepatology* 2007;45:921–8.
23 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402–8.
24 Silver N, Best S, Jiang J, *et al*. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol Biol* 2006;7:33.
25 Dahari H, Layden-Almer JE, Perelson AS, *et al*. Hepatitis C viral kinetics in special populations. *Curr Hepat Rep* 2008;7:97–105.
26 Neumann AU, Lam NP, Dahari H, *et al*. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998;282:103–7.
27 Honda M, Sakai A, Yamashita T, *et al*. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. *Gastroenterology* 2010;139:499–509.
28 Urban TJ, Thompson AJ, Bradrick SS, *et al*. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. *Hepatology* 2010;52:1888–96.
29 Dill MT, Duong FH, Vogt JE, *et al*. Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. *Gastroenterology* 2011;140:1021–31.
30 Hiraga N, Abe H, Imamura M, *et al*. Impact of viral amino acid substitutions and host interleukin-28b polymorphism on replication and susceptibility to interferon of hepatitis C virus. *Hepatology* 2011;54:764–71.

Clinical Infectious Diseases Advance Access published May 23, 2013

High Levels of HBV after the Onset Lead to Chronic Infection in Patients with Acute Hepatitis B

Hiroshi Yotsuyanagi[1,7], Kiyoaki Ito[2, 5, 7], Norie Yamada[1,3,4], Hideaki Takahashi[3], Chiaki Okuse[3], Kiyomi Yasuda[4], Michihiro Suzuki[3], Kyoji Moriya[1], Masashi Mizokami[2], Yuzo Miyakawa[6], and Kazuhiko Koike[1]

[1]Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Bunkyo, Tokyo, Japan; [2]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan; [3]Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki, Japan; [4]Department of Internal Medicine, Center for Liver Diseases, Kiyokawa Hospital, Suginami, Tokyo, Japan; [5]Department of Microbiology and Immunology, Aichi Medical University School of Medicine and [6]Miyakawa Memorial Research Foundation, Minato, Tokyo, Japan.

[7]*H.Y. and K.I equally contributed to this work*

Key point

In acute HBV infection, genotype A is associated with the development of persistent infection, presumably determined by positive HBsAg at 12 months from the onset. Persistent infection may be predicted by measuring HBsAg at 12 weeks from the onset.

FOOTNOTE

Address requests for reprints to: Hiroshi Yotsuyanagi, MD, Graduate Institute of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo, Tokyo 113-8655, Japan. e-mail: hyotsu-tky@umin.ac.jp; phone: 81-3-5800-8801, fax: 81-3-5800-8804.

Abstract

Background. Some patients with acute HBV infection develop chronic infection. However, the method for identifying them has not been established.

Methods. We followed 215 Japanese patients with acute HBV infection until the clearance of HBsAg or the development of chronic infection. Levels of HBsAg and HBV DNA were serially monitored from the onset.

Results. Of the 215 patients, 113 (52.5%) possessed HBV genotype A, 26 (12.0%) genotype B, and 73 (34.0%) genotype C. Twenty-one of the 215 (9.8%) developed chronic infection, with the persistence of HBsAg for > 6 months. The rate of chronicity of genotype A, B, and C was 12.4%, 3.8%, and 8.2%. Of the 21 patients, only six (2.8%) patients, including five with genotype A, failed to clear HBsAg within 12 months. Levels of HBsAg at 12 weeks and HBV DNA at 4 weeks were useful for distinguishing the patients who became chronic from those who did not (P <.001 and P < .001, respectively). Likewise, the levels of HBsAg at 12 weeks and HBV DNA at 8 weeks were useful for discriminating between the patients who lost HBsAg within 12 months and those who did not (P < .01 and P < .05, respectively).

Conclusions. In acute HBV infection, clearance of HBV may happen between 6 and 12 months from the onset. Only those who fail to clear HBV within 12 months from the onset may really develop chronic infection.

The clinical outcome of acute hepatitis B is self-limited in the majority of immunocompetent adults. However, some patients run a prolonged or even chronic course, or are complicated by acute liver failure. Several factors are implicated in different clinical courses.

Hepatitis B virus (HBV) genotypes and subtypes are known to influence the clinical outcome of acute hepatitis B. For instance, HBV subgenotype B1 is associated with fulminant hepatic failure in acute hepatitis B [1]. On the other hand, genotype A is associated with chronic sequellae [2-5]. Furthermore, patients with subgenotype C2 are more likely to develop chronic infection than those with subgenotype B2 [6]. These characteristics may reflect viral kinetics in acute HBV infection that would be different among HBV infections with distinct genotypes/subgenotypes, but little is known about them.

Quantitation of hepatitis B surface antigen (HBsAg), in addition to HBV DNA, has been introduced to analysis of viral kinetics in patients with chronic hepatitis B in recent years. HBsAg levels are useful, also, for estimating viral loads and predicting the response to antiviral treatments [7-9], and for figuring out the natural history of chronic hepatitis B [10, 11]. Therefore, HBsAg and HBV DNA would be instrumental in foretelling the outcome of acute hepatitis B. However, the clinical utility of these markers in patients with acute hepatitis B is largely unknown.

Therefore, the aim of the present study was to examine differences in viral kinetics among patients with acute hepatitis B, who were infected with HBV of different genotypes, and evaluate the usefulness of quantifying HBsAg and HBV DNA for predicting the clinical outcome.

PATIENTS AND METHODS

Patients

This was a retrospective study of patients who were diagnosed as acute hepatitis B in our institutions during 1994 through 2010. Criteria for the diagnosis of acute hepatitis B were: (i) acute onset of liver injury without a previous history of liver dysfunction; (ii) detection of HBsAg in the serum; (iii) IgM antibody to HBV core (anti-HBc) in high titers (detectable in sera diluted 10-fold) [3]; (4) absence of a past or family history of chronic HBV infection; and (5) exclusion of co-infection with hepatitis A virus, hepatitis C virus or other hepatotropic viruses by serological tests. Among the 232 patients who met these criteria, 215 patients (159 men and 56 women with a mean age of 31.8 ± 10.0 years) whose serum samples were available for virological analyses were included in the study. No patient developed liver failure.

No patient received antiviral treatment. Of the 215 patients, 159 (74.0%) patients could be regularly followed up until the confirmation of clinical outcomes. Based on the duration of HBsAg (defined as the interval between the onset [defined by the first visit] and the last visit with detectable HBsAg), we classified the 159 patients into the following four groups (the duration of HBsAg indicated in parentheses): Group I (< 3 months); Group II (3–6 months); Group III (> 6–12 months); and Group IV (> 12 months). Changes in virological parameters were analyzed in relation with clinical characteristics.

The study was approved by the Ethics Committees of our institutions, and a written informed consent on the purpose of this study was obtained from each patient.

Quantification of Serological Markers for HBV Infection and HBV DNA

HBsAg had been measured quantitatively by the chemiluminescent enzyme-linked immunosorbent assay (Sysmex JAPAN Co., Ltd., Kobe, Japan) every 2–4 weeks, until the clinical outcome was known. It has a dynamic range of 0.03–2, 500 IU/mL. Sera