博士論文

Genetic Alterations of Hepatitis Virus Oncogenes in Association with Treatment Resistance and Carcinogenesis

（治療抵抗性と肝発癌に関与する肝炎ウイルス癌遺伝子変異）

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ABBREVIATIONS

AA	: amino acid
ALT	: alanine aminotransferase
APASL	: the Asian Pacific Association for the Study of the Liver
AsC	: asymptomatic carrier
AST	: aspartate aminotransferase
BCP	: basal core promoter
BMI	: body mass index
C/EBP	: CCAAT/enhancing binding protein
cEVR	: completely early virological response
CHB	: chronic hepatitis B
CP	: core promoter
Enh2	: enhancer 2
EVR	: early virological response
GTP	: glutamyl transpeptidase
HBV	: hepatitis B virus
HCC	: Hepatocellular carcinoma
HCV	: hepatitis C virus
HNF3	: hepatocyte nuclear factor 3
IC	: inactive carriers
LC	: liver cirrhosis
NRE	: negative regulatory element
NT	: Nucleotide

NVR : null response

ORF : open reading frame

PEG-IFN : Peglated Interferon

PLT : platelets

PR : Partial virological response or partial non-response

RBV : Ribavirin

SNP : single nucleotide polymorphism

SOC : standard of care

SVR : sustained virological response

Tbil : total Bilirubin

UTRs : untranslated regions

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PREFACE

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second leading cause of cancer death worldwide [1]. Approximately 782,000 new HCC cases and 746,000 cancer deaths occurred in 2012 [2]. HCC usually accompanies no obvious symptoms until develops to late stages, and currently there exists no ideal predictive biomarker/methods for early-stage diagnosis. Moreover, the available treatments are not tumor-specific and not very effective either when disease progresses to late stages. Herein the prognosis for HCC is quite poor with the mortality to incidence rate ratio reaching 0.95, and HCC remains to be a major health problem worldwide.

The main difficulty for developing ideal biomarkers for HCC early-stage diagnosis may be because of the fact that HCC is a multi-factor and multi-step process. A variety of risk factors such as viruses, alcohol, and aflatoxin have been associated with HCC development. However, the strongest link comes from the chronic infection of hepatitis B virus (HBV) and/or hepatitis C virus (HCV) which constituted around 80% of HCC cases, 53% and 25% of which are by HBV and HCV, respectively (Figure 1). A unique disease progression usually experiences several stages from chronic hepatitis virus infection to HCC, namely, asymptomatic carrier, chronic hepatitis, fibrosis, liver cirrhosis, and HCC, successively, while the actual prognosis varies and HCC can occur at any stage of chronic liver diseases. Multiple factors such as host factors, viral factors, and therapeutic intervention play roles in determining the final prognosis. It is estimated that approximately 30% of chronic HBV carriers and 50% of HCV carriers will develop progressive chronic liver diseases including HCC within 20-30 years [3]. Appropriately personalized medical intervention strategies are therefore highly desired. Although intensive investigations have

been conducted in this field, details in the pathogenic modes of genetic alterations in viral genes for hepatocarcinogenesis still need to be clarified.

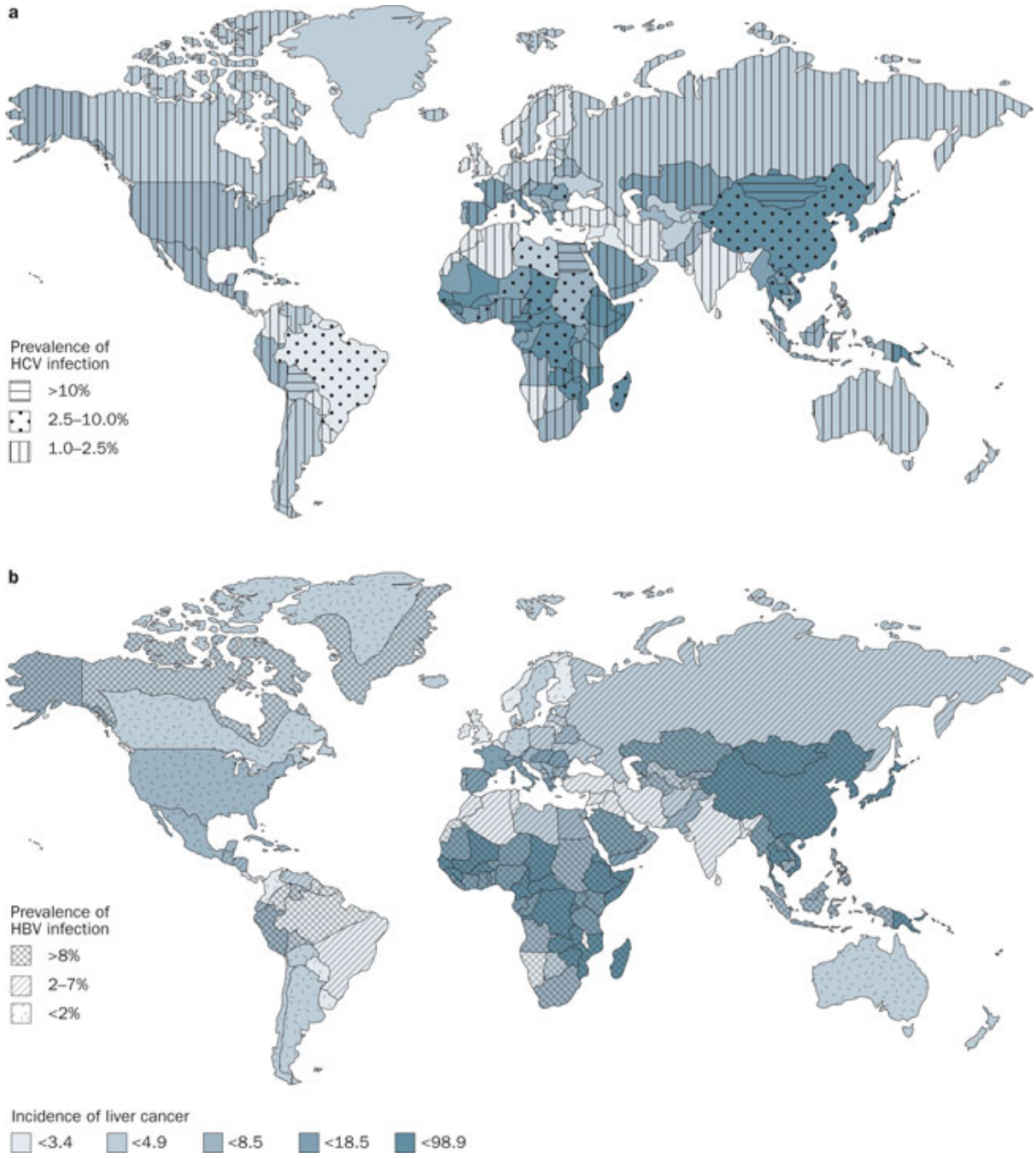


Figure 1. Global prevalence of chronic HCV infection and chronic HBV infection, and different incidence rates of HCC. The geographic distribution of chronic HCV infection (a) and chronic HBV infection (b). In areas where have a relatively high prevalence of HBV and HCV, the occurrence of HCC is also elevated **(**Source**:** Yang JD et al. *Nat Rev Gastroenterol Hepatol.* 2010).

HBV belongs to Hepadnaviridae family, and is a partially double-stranded circular DNA virus with the length at 3.2 kb. The small viral genome contains four overlapping open reading frames (ORFs: S, C, P, and X), encoding HBs, HBc/HBe, Polymerase, and HBx protein, respectively (Figure 2). HBV replicates via an RNA intermediate and also integrates into the host genome. With regard to HCV, it seldom integrates into host genome and exists mainly as an episome. HCV is a positive single-stranded RNA virus belonging to Flaviviridae family. The 9.6 kb linear viral genome contains a single large ORF, which could be translated into a polyprotein and then post-translationally cleaved into at least 10 polypeptides including three structural (core, E1, and E2) and multiple NS proteins (NS1-NS5) (Figure 3).

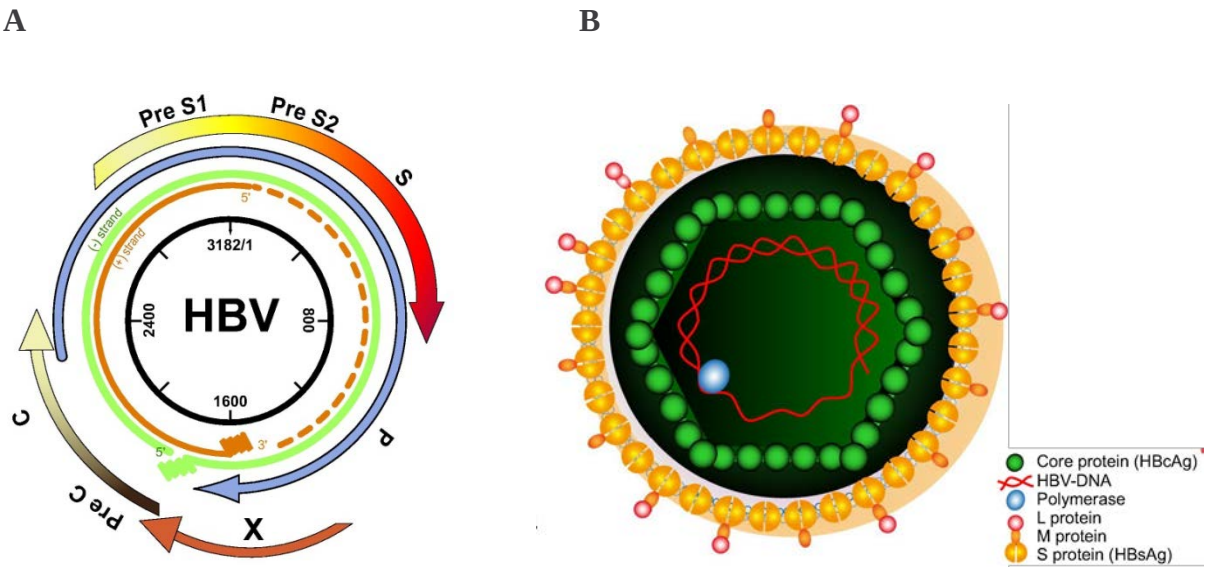
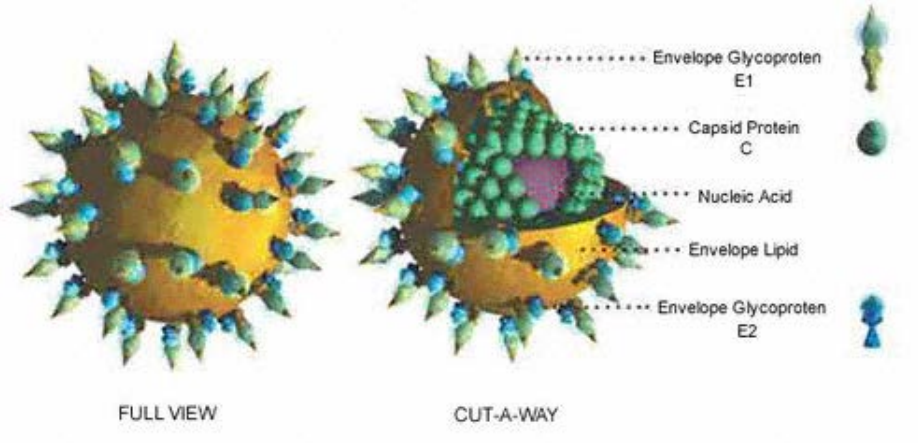


Figure 2. Structure of hepatitis B virus. (A) HBV is a partially double-stranded circular DNA virus with the length at 3.2 kb. The small viral genome contains four overlapping open reading frames (ORFs: S, C, P, and X), encoding HBs, HBc/HBe, Polymerase and HBx proteins. (B) The general property of HBV particles. The DNA genome is packaged with HBcAg and Polymerase, enclosed by HBsAg (Source: Teresa Pollicino et al, *Journal of Hepatology*, 2014).

A



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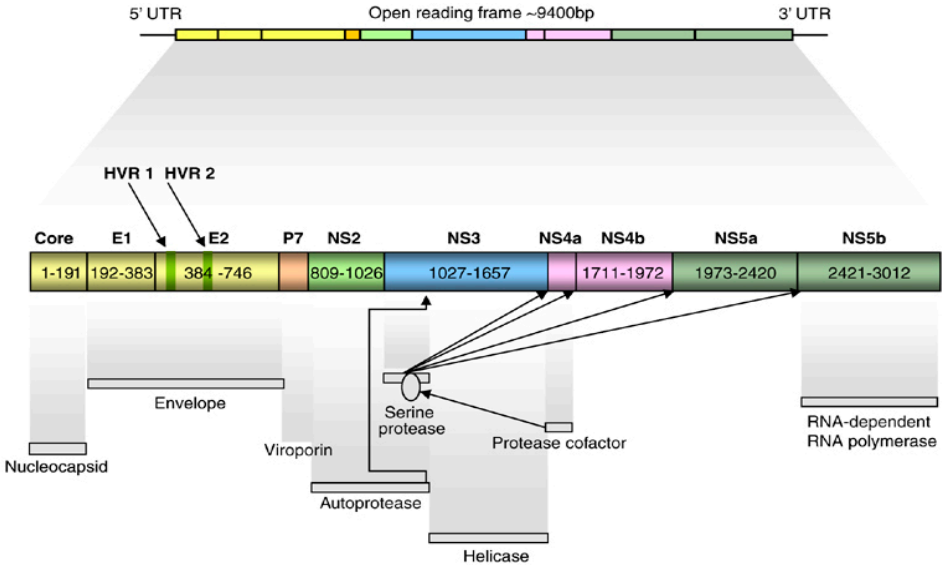


Figure 3. Structure of hepatitis C virus. HCV is a small (55–65 nm in size), enveloped, positive-sense single-stranded RNA belonging to the Hapacivirus genus within the Flaviviridae family. HCV RNA encodes a polyprotein composed of about 3,000 amino acids. The core protein, and two envelope proteins are classified as structural protein, while NS2, NS3, NS4A, NS4B, NS5A, and NS5B are non-structural proteins. (Source: A: Louis E Henderson et al, http://www.prn.org/; B: Andrew R Lloyd et al, *Immunology and Cell Biology*, 2007)

Despite the fact that HBV and HCV belong to different virus families and have distinct life cycles, still these two hepatitis viruses have similar properties in pathogenesis mechanisms. For instance, both HBV and HCV have been reported to play a direct role in the carcinogenesis via their viral proteins. In HBV, the most contributory protein

is HBx, encoded by the smallest open reading frame and reportedly related to carcinogenesis [4]. In HCV, it was believed that core protein played important roles in carcinogenesis [5]. Besides, both of the oncogenes are well conserved and contain multiple functional domains which facilitate their participation in a variety of cellular signaling pathways including those related to apoptosis, cell transformation, and cell cycle regulation [6]. Currently increasing amount of data have shown that certain genetic alterations in these two genes may affect not only the outcome of antiviral treatment but also hepatocarcinogenesis. However the results from multiple studies remain contradictory and the details still remain to be elucidated.

Here we focused our attention on the two viral oncogenes and investigated: 1) HCC-characteristic genetic alterations in HBV X region via a large scale retrospective study based on global data; 2) the association of HCV quasispecies which carried a specific mutation in core gene with antiviral treatment responses. The extended analyses of genetic alterations will help to identify molecular markers for liver cancer prevention, diagnosis, and treatment of HBV- and HCV- associated liver cancers.

CHAPTER 1

The Characteristic Changes in Hepatitis B virus X region for Hepatocellular Carcinoma: a Comprehensive Analysis Based on Global Data

ABSTRACT

Objectives: Mutations in hepatitis B virus (HBV) X region (HBx) play important roles in hepatocarcinogenesis while the results remain controversial. We aimed to uncover potential hepatocellular carcinoma (HCC) characteristic mutations in HBx from patients infected with HBV genotype C and their distribution in different disease phases and genotypes.

Methods: HBx sequences from an online global HBV database were screened and classified into Non-HCC or HCC group using diagnosis information. Data of patient age, gender, country or area, and viral genotype were also extracted. The effects of mutations on HCC risk were evaluated by logistic regression.

Results: 1) Full length HBx sequences (HCC: 161; Non-HCC: 954) originated from 1115 human sera across 29 countries/areas were extracted from the downloaded 5956 HBx sequences. Genotype C accounted for 40.6% (387/954) of Non-HCC and 89.4% (144/161) of HCC. 2) Between genotype C HCC and Non-HCC, significantly different distributions were observed at 16 nucleotide positions. 3) Logistic regression exhibited that mutations A1383C (OR: 2.32, 95% CI: 1.34-4.01), R1479C/T (1.96, 1.05-3.64; 5.15, 2.53-10.48), C1485T (2.40, 1.41-4.08), C1631T (4.09, 1.41-11.85), C1653T (2.58, 1.59-4.19), G1719T (2.11, 1.19-3.73), and T1800C (23.59, 2.25-247.65) were independent risk factors for HCC related to genotype C HBV, presenting different trends among individual disease stages. 4) Several risk mutations for genotype C HCC pre-existed, even as major types, in other genotypes at early disease phases.

Conclusions: Mainly located in HBx transactivation domain, viral promoter, protein/miRNA binding sites, and the area for immune epitopes were mutations associated with HCC risk. The signatures of the mutations were unique to disease phases leading to HCC, indicative of molecular counteractions between the virus and host in hepatocarcinogenesis.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection constitutes a major health problem affecting more than 400 million people earthwide. Advanced liver diseases including liver cirrhosis (LC) and hepatocellular carcinoma (HCC) are induced by persistent HBV infection. This is true for around half of the HCC cases globally and even up to 90% in the area where HBV is highly prevalent [7]. It usually takes two to three decades for asymptomatic HBV carrier (AsC) to develop HCC, while outcomes of patients varied considerably according to the balance between host immune system and the virus.

The HBV genome is made of circular DNA, which is partially double-stranded and 3.2 kb long in length. Four overlapping open reading frames (ORFs) are contained on that: P, C, S, and X, encoding polymerase, core and HBe antigen, surface antigen, and HBx protein, respectively. Four viral promoters, preS1, preS2, core and X, and two enhancers, Enh 1 and Enh 2, overlapping with the ORFs, control transcription of those proteins. HBV genome has a mutation ratio approximately $1.4\text{-}3.2\times10^{-5}$ nucleotide (nt) mutations per site per year, which is higher than other DNA viruses due to the proof-reading deficiency of the viral reverse transcriptase. Moreover, the endogenous and exogenous pressures, represented by host immunity and therapeutic intervention by antiviral drugs and vaccines, respectively, render the viral mutations more complicated, resulting in the formation of various HBV genotypes.

HBV genome is categorized into at least 9 genotypes (A-I) based on the difference larger than 8% of the whole genome sequences. Geographical distributions of the virus are distinct among different genotypes, and various clinical outcomes are induced accordingly. For instance, genotype C, the most prevalent one in Asia, was demonstrated to be more associated with HCC than genotype B [8,9]. Also genotype D prevalent in Africa, Europe, the Mediterranean region and India and genotype F prevalent in Central and South America were

proved to be more carcinogenetic in local cohorts [10] (Figure 4). Certain HBx mutations in specific HBV strains have been displayed to be critical for severe liver diseases including HCC [11,12,13,14,15]. The results remained inconsistent, however, even in the same genotypes or cohorts with similar ethnic backgrounds. This could be ascribed to the limited nucleotides/patients and cohorts investigated. Currently, it still remains obscure whether there were mutations universally responsible for HCC and whether HCC risk mutations exist only in certain genotypes. Here we explored characteristic mutations in genotype C HBx for HCC and examined their distribution among different disease phases and HBV genotypes, exploiting a global HBV database.

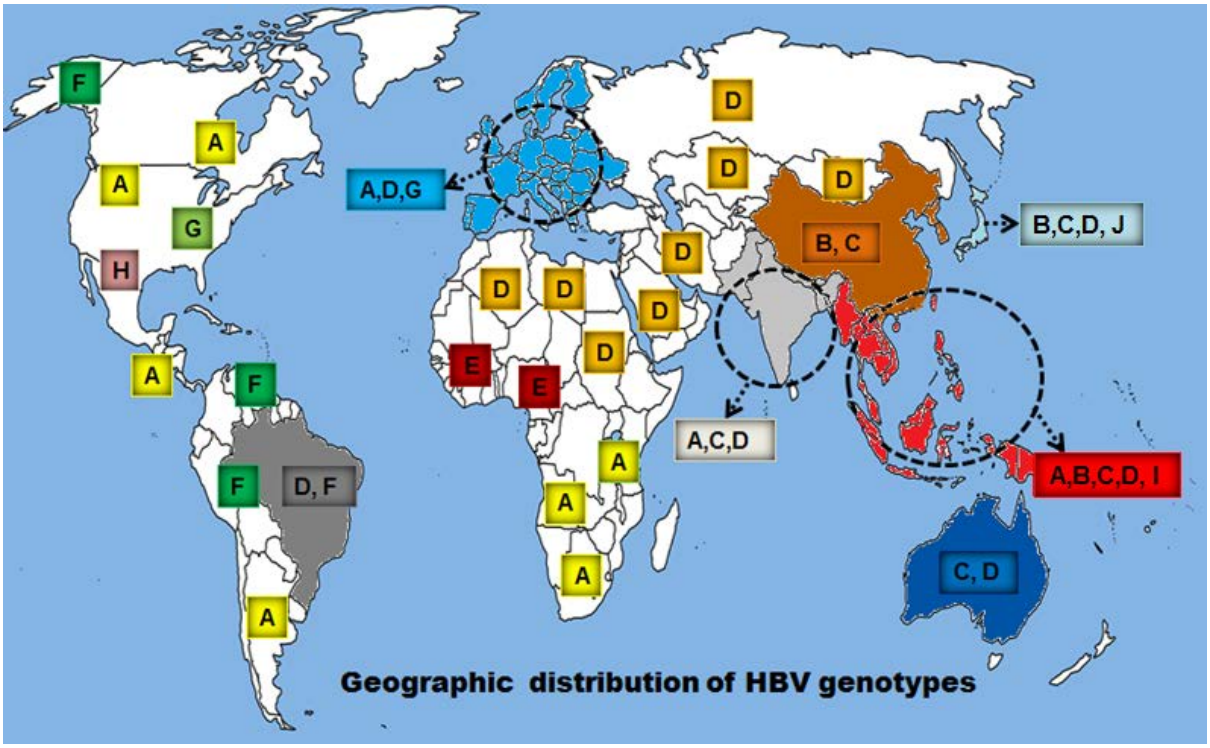


Figure 4. Geographic distribution of HBV genotypes worldwide. (Source: Zahid Hussain et al, "*Practical Management of Chronic Viral Hepatitis*" Chapter 2.)

MATERIALS AND METHODS

Sequences collection

HBx sequences were downloaded from Hepatitis Virus Database (HVDB, http://s2as02.genes.nig.ac.jp/), a user-friendly online public nucleotide database focused on hepatitis viruses especially HBV and HCV. All hepatitis virus sequences deposited in HVDB were retrieved from DNA Data Bank of Japan (DDBJ) and all of the information was updated from DDBJ periodically. The version we exploited was DDBJ Rel. 95 in Dec 2013, which contained 5956 nucleotide sequences of HBV X region. All the formats of sequences were in conformity to Genbank.

Sequences screening

Sequences multi-alignment

Sequences multi-alignment was firstly performed using ClustalW online analysis (DDBJ, http://ddbj.sakura.ne.jp/searches-e.html), results were examined manually twice.

Sequences exclusion criteria

We screened the sequences by attached information in HVDB database and sequence related publications in Pubmed (http://www.ncbi.nlm.nih.gov/pubmed) successively: publication, origin, diagnosis etc. Briefly, we set exclusion criteria as 1) since the attached information of database sequences were very limited, it's impossible to distinguish the origin of all the sequences and the diagnosis of patients with HBV infection when publications concerned are unavailable. Therefore sequences with no any published information were excluded first; 2) since virus sequences from different origins may varied a lot, we next excluded sequences from Non-human origins and those from liver tissue or cell lines; 3) Sequences will be excluded if the related paper did not specify the diagnosis clearly. For instance, if a paper only stated that their sera samples were from patients with chronic HBV infection, all related sequences will be excluded; 4) When more than one sequence were from same patient, either from same or different time point, only one sequence with available information would be

used; 5) Sequences from patients with acute disease phases, co-infection with other viruses, or complications were excluded; 6) Sequences with recombinant HBV genotypes were excluded; 7) In chronic HBV infected patients, the deletion and insert of HBV sequences are also frequent events. Here we excluded sequences with insertions or deletions in order to focus on mutations in complete sequences.

Sequence inclusion criteria

Sequences finally enrolled are: 1) Full length HBV X sequence (465 bp, from 1 at A of ATG and to 465 at A of TGA/TAA, or G of TAG); 2) human sera origin; and 3) with diagnostic information and classified into non-HCC or HCC (Figure. 5). Information of patient age, gender, country or area, and viral genotype were also extracted. Viral genotypes were also confirmed by online NCBI genotyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).

Statistical analyses

Continuous data were expressed as Mean (range) and compared by t-test. Categorical data were analyzed by Fisher's exact test (SPSS 16.0). $P<0.05$ was considered to have significant differences. Logistic regression was performed to evaluate the effects of mutations on HCC risk.

Ethics statement

According to Ethical Guidelines for Clinical Research (2008, MHLW), this study protocol was exempted from obtaining approval from our ethics committee since data used for analyses in this retrospective study were all from published papers and a public database. In addition, we used only anonymized and de-identified patients' data with approval of each ethics committee/institutional review board in accordance with the declaration of Helsinki.

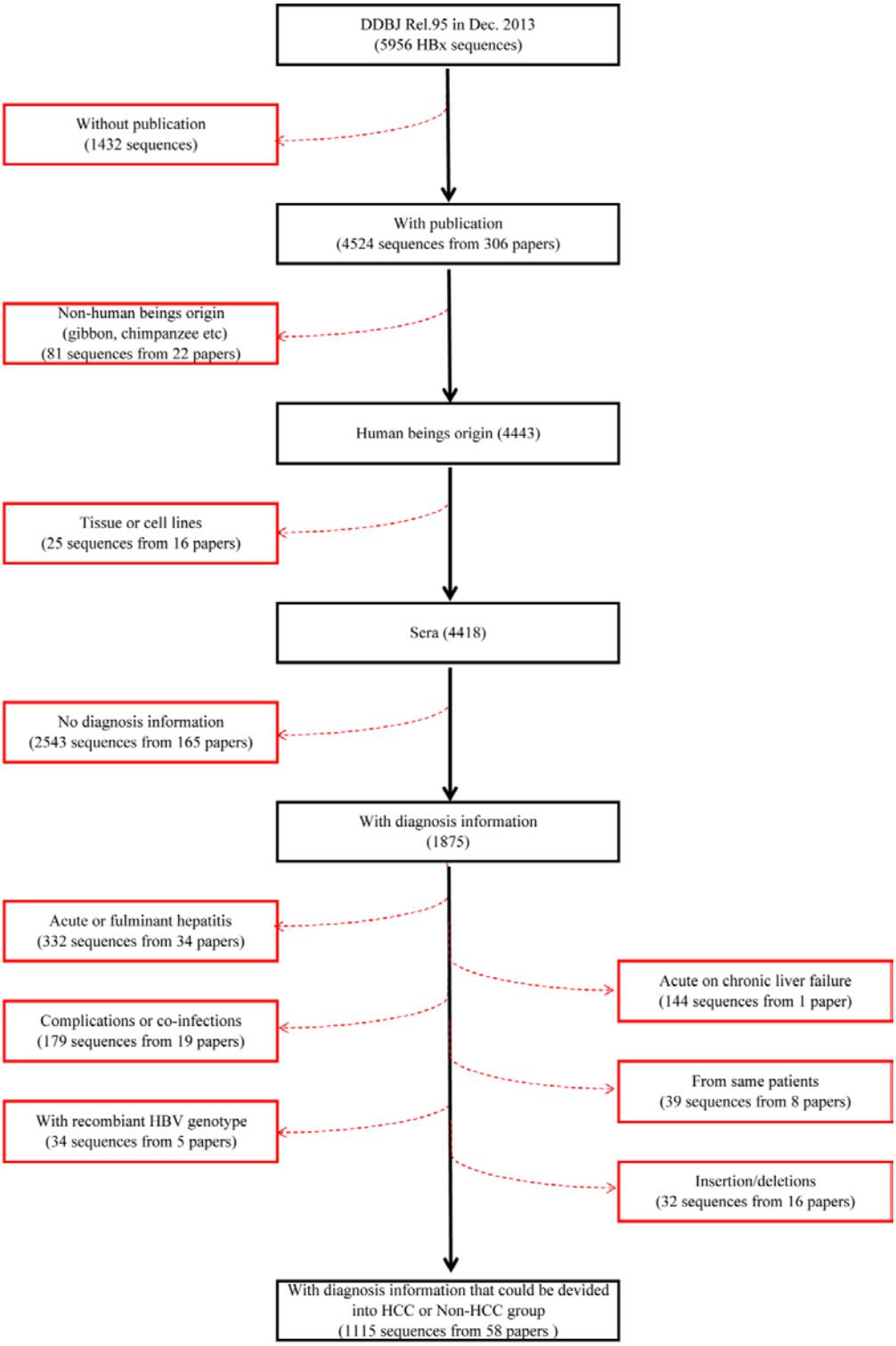


Figure 5. Flowchart for screening HBx sequences downloaded from an online global database. HBx sequences were downloaded from Hepatitis Virus Database (HVDB, http://s2as02.genes.nig.ac.jp/). The version we exploited was DDBJ Rel. 95, containing 5956 HBx sequences in total. Sequences were then screened successively by attached information such as publication, origin, and diagnosis. Sequences enrolled should be 1) Full length HBV X sequence; 2) human sera origin; and 3) with diagnosis information and thus could be classified to non-HCC or HCC group. Finally 1115 full length HBx sequences (HCC, 161; and Non-HCC, 954) from 58 publications were extracted for further analyses.

RESULTS

Enrolled HBx sequences

From the downloaded 5956 HBV X sequences, 1115 HBx sequences (HCC, 161; and Non-HCC, 954) covering 29 countries/areas were finally extracted (Table 1). Non-HCC included five subgroups with different disease phases: AsC, inactive carriers (IC), chronic hepatitis B (CHB), LC and HBV-related chronic liver diseases such as CHB and LC without HCC. Genotype C accounted for 40.6% (387/954) of Non-HCC and 89.4% (144/161) of HCC. Of Non-HCC sequences, 52.7% was accounted for by genotype B, D, and E. We then compared the age and gender of the patients between the two groups according to the information available. The average age in the HCC group was 10 years older than that in the Non-HCC group ($P<0.01$) and the ratio of males was higher in HCC (42/45, 93.3%) than in Non-HCC (99/128, 77.3%) ($P=0.02$). In Table 2, the demographic information of the enrolled 531 genotype C sequences was summerized. Genotype C Non-HCC group consisted of four subgroups including AsC (18), CHB (38), LC (27) and HBV-related chronic liver diseases such as CHB and LC without HCC (304). The majority of both genotype C Non-HCC group (79.8%) and HCC group (97.9%) were constituted of sequences from Japan, Mainland China and South Korea.

Table 1. The demographic information of enrolled sequences.

Group		HCC	Non-HCC	P value[a]
Number		161	954	
Gender	Known (M/F)	45 (42/3)	128 (99/29)	0.02
Age	Mean (n/range)	56.3 (46/26-89.8)	46.0 (130/5-86)	<0.01
Diagnosis	HCC	161 (100%)	/	/
	AsC	/	366 (38.4%)	/
	IC	/	27 (2.8%)	/
	CHB	/	118 (12.4%)	/
	LC	/	71 (7.4%)	/
	Unclear but no HCC	/	372 (39.0%)	/
Countries/Areas	Australia	0.6%	0.3%	/
	Belgium	/	2.1%	/
	Bolivia	/	0.7%	/
	Brazil	/	0.1%	/
	Cameroon	/	0.5%	/
	Chile	/	2.2%	/
	France	/	0.1%	/
	Ghana	/	1.5%	/
	Guinea	/	8.2%	/
	Hong Kong	1.2%	4.9%	/
	India	/	5.5%	/
	Indonesia	1.2%	0.2%	/
	Iran	/	8.0%	/
	Ireland	/	0.1%	/
	Japan	67.1%	11.0%	/
	Mainland China	12.4%	25.0%	/
	Malaysia	/	3.1%	/
	Niger	/	1.9%	/
	Nigeria	/	4.9%	/
	Philippines	3.1%	0.9%	/
	Serbia	/	0.5%	/
	South Africa	/	1.8%	/
	South Korea	14.3%	4.2%	/
	Spain	/	0.1%	/
	Taiwan	/	0.7%	/
	Thailand	/	0.1%	/
	Turkey	/	10.5%	/
	Uzbekistan	/	0.6%	/
	Vietnam	/	0.1%	/
Genotypes	A	2	34	/
	B	14	76	/
	C	144	387	/
	D	/	268	/
	E	/	159	/
	F	/	27	/
	H	/	2	/
	I	1	1	/

[a]Differences as proportions have been presented where calculable. HCC, hepatocellular carcinoma; AsC, asymptomatic HBV carriers; IC, inactive HBV carriers; CHB, chronic hepatitis B; LC, liver cirrhosis.

Table 2. The demographic information of enrolled genotype C sequences.

Group		HCC	Non-HCC	P value[a]
Number		144	387	
Gender	Known (M/F)	42 (39/3)	61 (52/9)	0.35
Age	Mean (n/range)	56.9 (43/35.9-89.8)	48.6 (61/5-74.3)	<0.01
Diagnosis	HCC	144 (100%)	/	/
	AsC	/	18 (4.7%)	/
	IC	/	/	/
	CHB	/	38(9.8%)	/
	LC	/	27 (7.0%)	/
	Unclear but no HCC	/	304 (78.6%)	/
Countries/Areas	Australia	0.7%	0.3%	/
	Belgium	/	0.3%	/
	Bolivia	/	0.3%	/
	Brazil	/	0.3%	/
	Cameroon	/	/	/
	Chile	/	/	/
	France	/	/	/
	Ghana	/	/	/
	Guinea	/	/	/
	Hong Kong	/	11.6%	/
	India	/	/	/
	Indonesia	/	0.3%	/
	Iran	/	/	/
	Ireland	/	/	/
	Japan	73.6%	22.0%	/
	Mainland China	8.3%	47.5%	/
	Malaysia	/	3.4%	/
	Niger	/	/	/
	Nigeria	/	/	/
	Philippines	1.4%	1.8%	/
	Serbia	/	/	/
	South Africa	/	/	/
	South Korea	16.0%	10.3%	/
	Spain	/	/	/
	Taiwan	/	1.0%	/
	Thailand	/	0.3%	/
	Turkey	/	0.3%	/
	Uzbekistan	/	0.3%	/
	Vietnam	/	0.3%	/

[a]Differences as proportions have been presented where calculable. HCC, hepatocellular carcinoma; AsC, asymptomatic HBV carriers; IC, inactive HBV carriers; CHB, chronic hepatitis B; LC, liver cirrhosis.

Difference in genotype C nucleotide distribution between Non-HCC and HCC

Next we examined each nucleotide position of genotype C HBx sequences between the two groups, genotype C HCC (144) and genotype C Non-HCC (387). Sixteen out of all 465 positions exhibited significant differences in Table 3. In overlapping cis-elements, four in Enh2 region (nt1636-1744) and nine in core promoter (CP) region (nt1613-1849) were identified. Also the mutant ratios of five positions (A1383C, R1479Y, C1485T, C1653T, and G1719T) were demonstrated to be higher in HCC group by more than 10% than in Non-HCC group.

Table 3. Sixteen HBx nucleotide positions showed significant differences between HBV genotype C infected patients with and without HCC by univariate analysis.

Nucleotide changes			Nucleotide location in X region	Ratio in groups		P value
				Non-HCC	HCC	
A	→	C	nt 1383	**25.8%**	**52.8%**	<0.001
C	→	T	nt 1425	10.1%	17.4%	0.009
T	→	C	nt 1458	95.9%	100.0%	0.009
R	→	Y	nt 1479	**30.5%**	**64.6%**	<0.001
C	→	T	nt 1485	**16.8%**	**29.9%**	0.003
G	→	A	nt 1511	7.0%	13.9%	0.028
G	→	A	nt 1569	0%	0.7%	0.018
G	→	A	nt 1630	81.4%	91.0%	0.012
C	→	T	nt 1631	1.8%	8.3%	0.001
C	→	T	nt 1653	**18.6%**	**35.4%**	<0.001
A	→	T	nt 1689	0.5%	2.8%	0.049
G	→	T	nt 1719	**57.6%**	**82.6%**	<0.001
A	→	G	nt 1721	85.3%	95.1%	0.002
A	→	G	nt 1757	94.6%	100.0%	0.005
G	→	A	nt 1775	86.3%	95.8%	<0.001
T	→	C	nt 1800	0.3%	3.5%	0.007

Positions that changed more than 10% were marked in bold. R: A or G; Y: C or T.

High risk mutations of HCC related to genotype C HBV

Subsequently the 16 point mutations were evaluated by multivariate analyses for exploring the risk mutations for genotype C HBV in relation to HCC. Logistic regression displayed that mutations A1383C (OR: 2.32, 95% CI: 1.34-4.01), R1479C/T (OR: 1.96, 95% CI: 1.05-3.64; OR: 5.15, 95% CI: 2.53-10.48), C1485T (OR: 2.40, 95% CI: 1.41-4.08), C1631T (OR: 4.09,

95% CI: 1.41-11.85), C1653T (OR: 2.58, 95% CI: 1.59-4.19), and G1719T (OR: 2.11, 95% CI: 1.19-3.73), T1800C (OR: 23.59, 95% CI: 2.25-247.65) were independent risk factors for genotype C HBV-related HCC (Table 4). Corresponding amino acid residues were also examined to find potential substitutions accompanied by those nucleotide mutations. We found six nonsynonymous (aa36, Thr → Pro; aa36, Thr → Ser; aa38, Pro → Ser; aa94, His → Tyr; aa116, Val → Leu; and aa143, Cys → Arg) and two synonymous substitutions (aa4, Arg → Arg; aa86, His → His) in HBx, and three synonymous substitutions in the overlapping polymerase (aa764, Leu → Leu; aa796, Gly → Gly; aa798, Tyr → Tyr). The structure and functions of HBx or the polymerase protein may be consequently altered by the substitutions (Figure 6).

Table 4. Seven nucleotide mutations of HBx sequences were independent risk factors for genotype C HBV-related HCC by multivariate analysis.

Mutations	Nucleotide location		Amino acid location		Mutant ratio in groups (%)		OR	P
	X	Cis-elements	HBx	Polymerase	Non-HCC	HCC	(95% CI)	value
AGG → **C**GG	nt1383	miRNA binding site	4 (Arg, Arg)	764 (Leu, Leu)	25.8	52.8	2.32 (1.34, 4.01)	0.003
RCT → **C**CT	nt1479	B cell epitope	36 (Thr, Pro)	796 (Gly, Gly)	22.7	49.3	1.96 (1.05, 3.64)	0.034
TCT			36 (Thr, Ser)	796 (Gly, Gly)	7.8	15.3	5.15 (2.53, 10.48)	< 0.001
CCG → **T**CG	nt1485	B cell epitope	38 (Pro, Ser)	798 (Tyr, Tyr)	16.8	29.9	2.40 (1.41, 4.08)	0.001
CA**C** → CA**T**	nt1631	CP, NRE	86 (His, His)		1.8	8.3	4.09 (1.41, 11.85)	0.01
CAT → **T**AT	nt1653	Box α, CP, C/EBP, Enh2	94 (His, Tyr)		18.6	35.4	2.58 (1.59, 4.19)	< 0.001
GTG → **T**TG	nt1719	BH3-like motif, CP, Enh2, HNF3, T cell epitope	116 (Val, Leu)		57.6	82.6	2.11 (1.19, 3.73)	0.01
TGC → **C**GC	nt1800	CP	143 (Cys, Arg)		0.3	3.5	23.59 (2.25, 247.65)	0.008

B cell epitope: region (aa positions 29-48); BH3-like motif: region (aa positions 116-132); Box α, region (nt1646-1668); C/EBP, CCAAT/enhancing binding protein, region (nt1643-1658); CP, core promoter, region (nt1613-1849); Enh2: enhancer 2, region (nt1636-1744); HNF3, hepatocyte nuclear factor 3, region (nt1713-1723); NRE, negative regulatory element, region (nt1611-1634); T-cell epitope: region (aa positions 116-127).

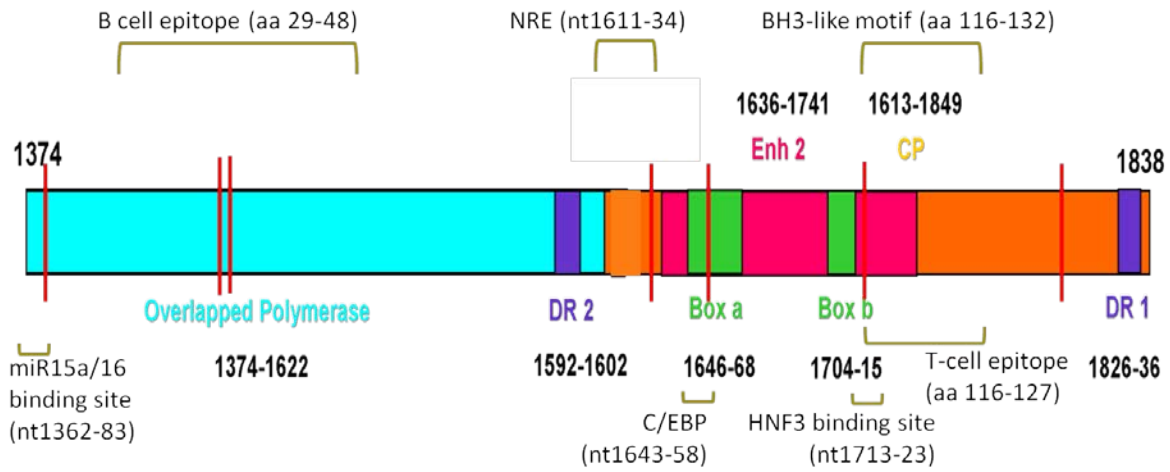


Figure 6. Locations of 7 genotype C HCC suspect mutations in HBx. Mutations associated with HCC risk were mainly located in HBx transactivation domain, viral promoter, protein/miRNA binding sites, and the area for immune epitopes.

Distribution of risk mutations for genotype C HBV-related HCC in different disease phases

In order to see the trend of those mutations in the progression to HCC, we investigated their distributions in genotype C sequences across different disease phases, AsC (18), CHB (38), LC (27), and HCC (144) (Figure 7). Since the double basal core promoter mutations (BCP) 1762T/1764A were reported to be associated with HCC [16][17], these two positions were also included in analyses though their ratios did not differ significantly between HCC and Non-HCC either in our univariate or multivariate analyses. As a result, several interesting characters were found: 1) except three mutations, 1479T, 1631T, and 1800C, all the mutations pre-existed in AsC, among which 1383C and 1719T were most pronounced. More than 50% of AsC possessed either one of these two mutations and 42% of AsC possessed both; 2) the ratios of four mutations, 1485T, 1631T, 1762T, and 1764A, demonstrated the increasing trend accompanying the disease progression; and 3) ratios of four mutations, 1383C, 1479C, 1479T, and 1719T, fluctuated in different disease phases.

In natural history of chronic HBV infection, HBeAg seroconvertion (HBeAg+/Anti-HBe- changes to HBeAg-/Anti-HBe+) has been considered as an important reference for clinical management. It was associated with lower HBV-DNA levels and clinical improvement of liver disease in the majority of patients [18,19,20]. Spontaneous HBeAg seroconversion is possible while the ratio is relatively low in those who infected with HBV at their early ages [21]. Whether there was a difference in distribution of genotype C HCC suspected mutations between HBeAg (+) AsC and HBeAg (-) AsC is an interesting question. We hence examined the HBeAg status of the 18 AsC by referring to published sequences. There were seven HBeAg (+) AsC, three HBeAg (-) AsC and eight AsC with unknown HBeAg status. We could not find significant differences in distributions those mutations between HBeAg (+) and HBeAg (-) AsC though 1653T tended to have a higher frequency in HBeAg (-) AsC (HBeAg+ AsC, 0%; HBeAg- AsC, 66.7%. P=0.06) (Figure 8). No significant changes were found when either HBeAg (+) AsC or HBeAg (-) AsC was compared with CHB.

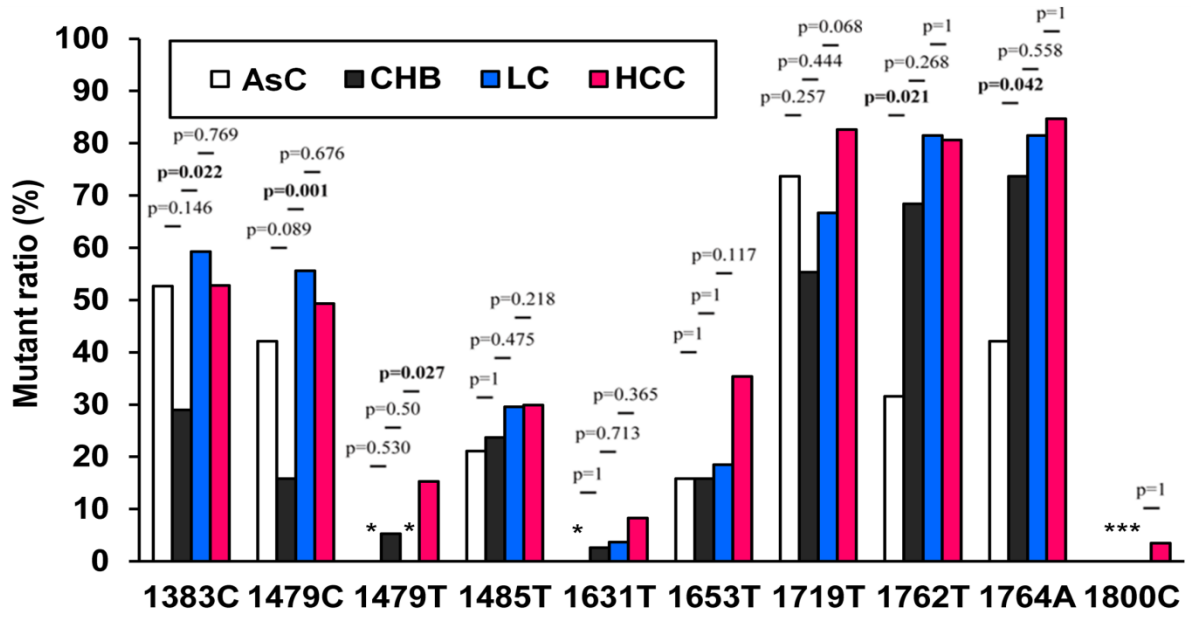
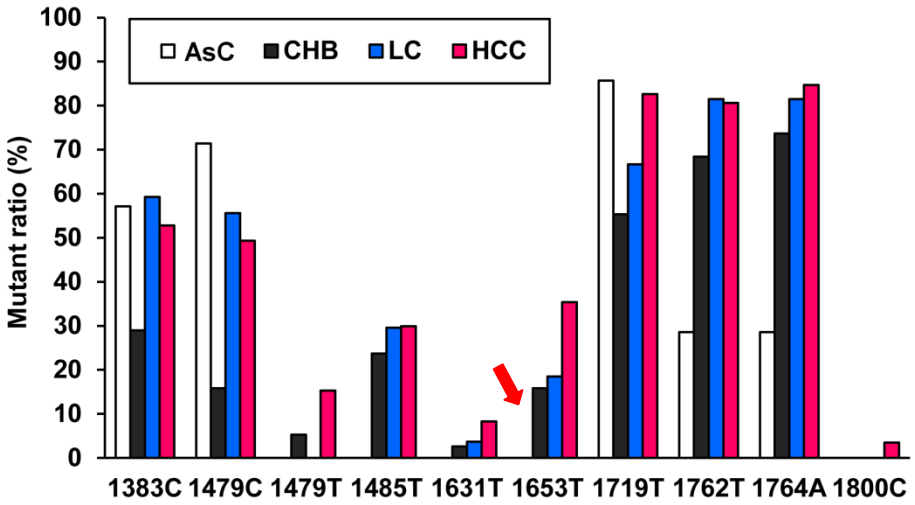


Figure 7. HCC risk mutations in genotype C sequences across different disease phases. The distribution of HBV genotype C HCC risk mutations among sequences from different disease phases (AsC, 18; CHB, 38; LC, 27; HCC, 144). Those mutations showed characteristics among different phases: 1) except three mutations (1479T, 1631T, and 1800C)

all the other mutations pre-existed in AsC, among which 1383C and 1719T were most pronounced. More than 50% AsC possessed either one of these two mutations and 42% AsC possessed both; 2) the ratios of four mutations (1485T, 1631T, 1762T, and 1764A) showed the increasing trend accompanied with the disease progression; and 3) ratios of 4 mutations (1383C, 1479C, 1479T, and 1719T) fluctuated among different disease phases. No mutants were observed in the cases denoted by asterisks.

A



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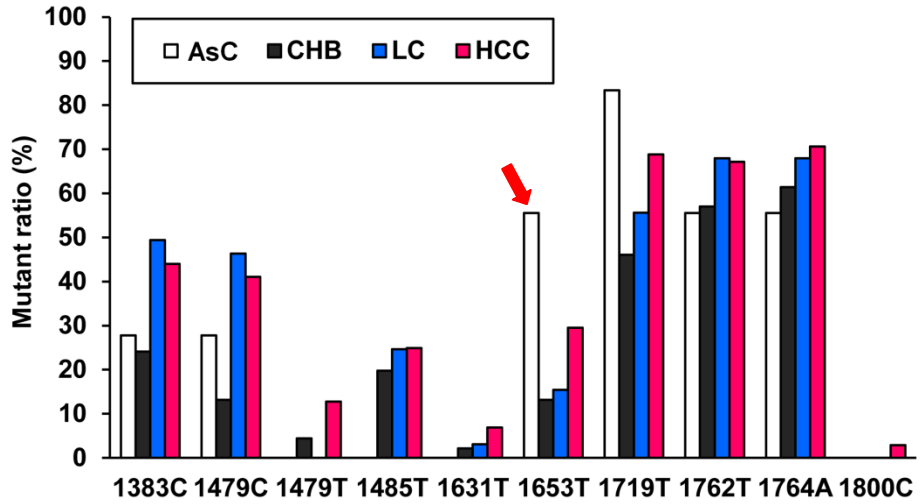


Figure 8. HCC risk mutations in genotype C sequences across different disease phases including HBeAg (+) AsC (A) and HBeAg (-) AsC (B). 1653T mutation (arrow in red) tended to have a higher ratio in HBeAg (-) AsC than HBeAg (+) AsC.

Distribution of risk nucleotides for genotype C HCC among different genotypes

In order to ask whether genotype C HCC risk mutations were commonly occurring in other genotypes, we checked the distribution of those mutations in our enrolled sequences. In AsC sequences (genotype A: 20, genotype B: 3, genotype D: 161, genotype E: 158, genotype F: 5, genotype I: 1), 100% of genotype A AsC presented 1485T and 90% presented 1631T, and 33.3% of genotype B AsC showed a mutantion C at nt1800 (Figure 9). In genotype A HCC sequences (n=2), two positions, 1485T and 1631T, showed genotype C HCC risk nucleotide. Moreover, genotype I HCC sequence (n=1) also presented two positions with genotype C HCC risk nucleotides, 1479C and 1800C. In genotype B HCC sequences (n=14), three positions, 1479T, 1631T and 1800C, showed the genotype C HCC risk nucleotides while they were at lower ratios less than 20% (Figure 10).

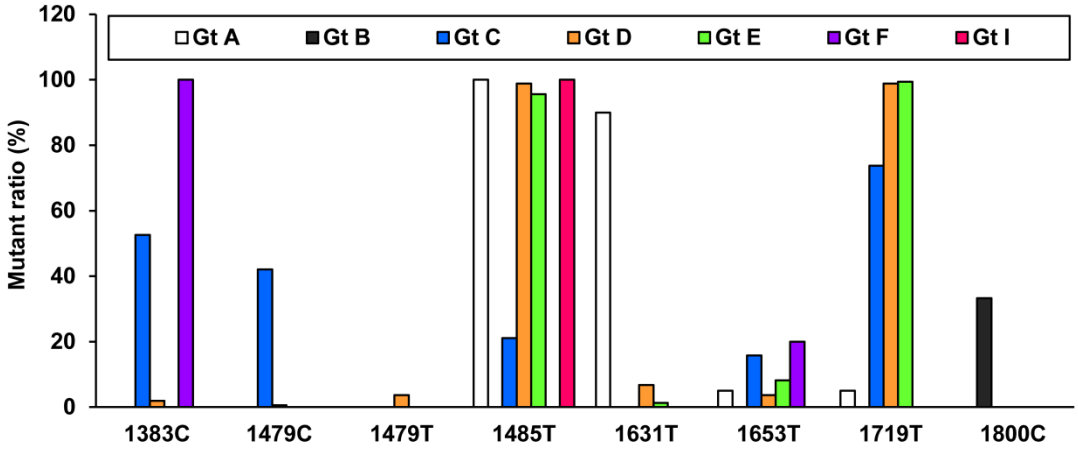


Figure 9. Frequency of HBV mutations in AsC with different genotypes. In AsC sequences with different genotypes (A, n= 20; B, n=3; D, n=161; E, n=158; F, n=5; I, n=1), 1485T and 1719T seemed to be prevalent. Gt: genotype.

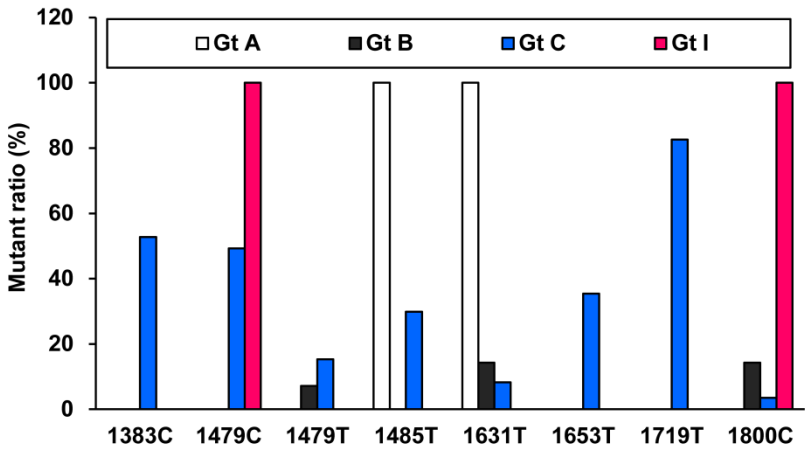


Figure 10. Frequency of HBV mutations in HCC with different genotypes. In HCC sequences with different genotypes (A, n= 2; B, n=14; C, n=144; I, n=1), more or less, some genotype C HCC risk nucleotides are presented.

DISCUSSION

HBV X region contains 465 bp (nt1374-1838) and encodes the 16.5 kDa HBx protein, partially overlapping with the RNase H domain of HBV Polymerase at the C terminus and containing critical cis-elements. Although the three-dimentional structure of HBx is still unknown, reportedly this unstructured protein could gain secondary structure under certain conditions and play roles via the interaction with target proteins [22]. Therefore genetic alterations in this region may affect not only the reading frame but also the overlapping cis-elements and possibly the target-binding affinities of HBx protein.

In recent years, increasing attention has been given to HBx mutations that potentially govern HBV related carcinogenesis, and only a few studies revealed certain mutations as risk factors for HCC. For instance, BCP mutations were frequently detected in HCC patients' sera infected with HBV [23,24,25,26]. Our lab also reported a single nucleotide mutation C1485T in HBx was HCC suspected mutation among Japanese patients infected with genotype C HBV [27]. As shown in Table 4, I could observe significant difference in the frequency of

mutation of nt1485 between Non-HCC and HCC (16.8% vs. 29.9%). However, studies reported so far are mainly based on patient samples that collected from local areas and the number of recruited patients is usually limited. Therefore, many contradictory results inevitably exist. To date, little is known about whether the impacts of those identified mutations are true also in other areas and genotypes.

The development of large-scale public database of viral sequence enabled us to investigate the HBV viral character in an overall perspective. In this study, based upon a global HBV sequence database, we compared HBx sequences between genotype C infected HCC and Non-HCC patients originated from different countries. Sixteen nucleotide differences between the two groups were found and seven of them (A1383C, R1479Y, C1485T, C1631T, C1653T, G1719T, and T1800C) were further identified to be critical for genotype C HBV related HCC. Three of them (R1479Y, C1631T and T1800C) were novelly identified in this study and 1383C, 1479C, 1653T and 1719T seemed to be genotype C unique mutation.

Nt1383 was located in the negative regulation domain of HBx (aa 1-50), and its 1383C mutation was first reported to be associated with HCC in a Korean cohort and was later found to induce a higher NF-kB activity in transformed cells [28,29]. In a Chinese clinical study, 1383C was also correlated with worse prognosis of patients after liver transplantation [30]. A group recently reported that microRNA 15a/16 (miR-15a/16), a tumor suppressor, directly targeted wild HBx RNA sequences (nt1362-1383) inducing Bcl-2 expression. The miR-15a/16 binding affinity declined significantly when mutations including one at nt1383 were introduced into the wild HBx [31]. Thus this mutation was suggested to prevent the infected cell from apoptosis by altering critical cell signaling pathways. In addition, it was speculated to regulate viral replication since the location was within miRNA

binding sites and competitively interfere with the interactions among host mRNAs, miRNAs, and viral RNAs [32].

Both nt1479 and nt1485 are located in B cell epitope region of HBx while the mutation frequency of nt1479 was higher than nt1485, which indicated that nt1479 may face higher immune pressure than did nt1485, though the mechanism still needs further clarification. Located in the transactivation domain of HBx (aa 51-140) associated with HCC due to the multiple cis-elements are Nt1631, 1653 and 1719 [33]. For example, nt1719 is located in a BH3-like motif (aa116-132) of HBx, through which HBx binds to CED-9, a homolog of Bcl-2 with the effects of pro-apoptosis [34]. *In vivo* and *in vitro* experiments showed that the binding affinity of HBx to CED-9 could be abolished by alternating residues in the BH-3 like motif of HBx [34,35]. Intriguingly, HBx could interact with two other Bcl-2 family members, Bcl-2 and Bcl-xL, via the same BH-3 like motif, and such interactions are critical for HBx to increase intracellular calcium concentration required for viral replication and cell death [36]. Thus, mutations emerging in this BH3-like motif can be carcinogenetic potentially affecting the interactions above.

When we analyzed the genotype C AsC sequences by different HBeAg status, we found that 1653T tended to have the higher emergence ratio in HBeAg- AsC than in HBeAg+ AsC (P=0.06). Previous studies had reported that this 1653T mutation was HCC suspected which is consistent with our study [8,37]. In addition, 1653T was correlated with acute exacerbation and liver failure of HBeAb + carriers [37,38,39]. It will be of great clinical interests if this mutation could behave as a marker for predicting disease progression of HBeAb+ patients though more supporting evidences based on large size of data is still needed.

The mutation at nt1800 is a novel HCC risk mutation discovered in our study. The function of the mutation in carcinogenesis remains unclear to date. However, a recent genome-wide analysis investigating HBV integration sites in 88 Chinese HCC patients

attracted our attention. The authors reported that almost 40% of the integrated HBV genomes were cleaved around at nt1800 [40]. Such a frequent breakage near a specific position implied potential roles of the site in carcinogenesis because HBV genome integration has been considered as an important factor in the HCC development. Thus, mutation at this position is thought to possibly affect the integration. In HBV chronic infection, moreover, it is currently believed that the genome integration is an early event. Thus, patients' sequences which possessed this mutation in AsC phase possibly posed a higher risk of HCC. From our data, we found that only genotype B AsC possessed a certain frequency of 1800C whereas other genotypes (A, C, D, E, F, and I) did not. In addition, several clinical studies revealed that genotype B tended to develop HCC more in younger patients than did genotype C but the mechanism was not yet understood [15,41]. This position suspected of integration may accelerate the carcinogenesis in patients infected with genotype B HBV.

In especially genotype C among several genotypes, the well-known BCP mutations (1762T1764A) were reported to be universal ones for risk of advanced liver diseases including HCC [23,24,42,43]. Meanwhile we could not find significant difference of BCP mutations between genotype C HCC and Non-HCC in our enrolled sequences. This could be explained by the pre-existence of BCP mutations in earlier disease phases, that is, that virus may have mutated shortly after the infection or that patients were initially infected with mutant type. We therefore inspected BCP mutation ratio in different disease phases and that was increased from AsC to CHB (1762T, AsC 33.3%, CHB 68.4%, P=0.02; and 1764A, AsC 44.4%, CHB 73.7%, P=0.04), while it seemed to reach the plateau from CHB phase (Figure 6). The differences between CHB and LC, and LC and HCC were not significant though the mutant ratio kept elevating over the disease progression. We conjectured that the two mutations might occur in the earlier stage of disease and act as a "driver mutation" or "first-hit" throughout the long period of carcinogenesis.

Similar trends were also found in other nucleotide positions. For instance, the ratio of 1485T among different disease phases (AsC, CHB, LC and HCC) was 21.1%, 23.7%, 29.6% and 29.9%, respectively. 1631T possessed 0% in AsC, 2.6% in CHB, 3.7% in LC and 8.3% in HCC. In addition, several other positions (4/465, data not shown) also presented similar trends though those positions have not been verified to increase HCC risk. Different from this format, the ratio of 1383C and 1479C/T fluctuated among disease phases. For instance, the mutant ratio of 1383C in genotype C AsC and HCC sequences were similar (AsC: 52.6%, HCC: 52.8%) while this site showed drastic changes from AsC to CHB, and CHB to LC (CHB: 28.9%, LC: 59.5%). 158 out of the whole 465 positions also showed the similar format. In addition, the remaining 301 nucleotide positions of HBx kept constant.

Many mutations occurring in HBV sequence during the treatment disappeared after withdrawal of antiviral treatment [25]. One group also reported that some nucleotide positions seemed to play inverse roles during the processions from CHB to LC and from LC to HCC [26]. In our study, by comparing HBx sequences among different disease groups, we found that the changes of HBx sequences could also be considered as a “multi-step and multi-factor” procession. During chronic infection, some positions kept conserved nucleotides or kept developing mutant nucleotides, i.e., nt1762, 1764, etc. We consider those positions may possess a constantly favored nucleotide type *in vivo*, but some positions changing their nucleotides across disease phases may possess a transiently favored type, as it were. For instance, HBV may produce new functional binding sites for molecules such as transcription factors and miRNAs or frame shifts by temporarily mutating some positions. The existing period of such type of mutation depends on the balance among host factors, microenvironments and viruses themselves.

Several genotype C HCC risk mutations preexisted in other genotypes. For instance, 1485T, which was first reported to be genotype C HCC risk factor by our lab in 2006 [27],

seemed to be very frequent in AsC with many genotypes (genotype A, D, E and I). genotype A possessed a ratio of 1485T for more than 90% either in AsC or HCC, but the ratios within genotype B AsC and HCC were zero. In addition, AsC with genotypes D and E exhibited high ratios for 1719T though we had no related HCC data. Besides, genotype B AsC showed the highest 1800C ratio among all the genotypes. It remains to be investigated whether these mutations in other genotypes were associated with the risk for HCC. Such distinct differences among genotypes may serve to develop prognostic tools in the future.

Collectively, nucleotides 1383C, 1479Y, 1485T, 1631T, 1653T, 1719T, and 1800C in HBV X region were independent risk factors for HCC in patients infected with HBV genotype C. But some of those also pre-existed in other HBV genotypes even as major types. Mutations associated with HCC risk were mainly located in HBx transactivation domain, viral promoter, protein/miRNA binding sites, and the area for immune epitopes. Moreover, the signatures of these mutations were unique to disease phases leading to HCC, indicating molecular counteractions between the virus and host during hepatocarcinogenesis. Further longitudinal studies are warranted to verify roles of these mutations in earlier disease stages and the process of oncogenesis with the interactive effects of other host factors encompassing age and gender and viral factors such as HBeAg status. Our study would facilitate early diagnoses and interventions for patients infected with genotype C HBV with the high risk of HCC.

CHAPTER 2

Amino Acid 70 Substitutions in Genotype 1b HCV Core Protein and Responses to PEG-IFN/RBV Treatment

ABSTRACT

Backgrounds: Hepatitis C virus (HCV) core protein amino acid 70 substitution [Arg (70 Wild) to Gln (70 Mutant)] is associated with PEG-IFN/RBV treatment failure and hepatocarcinogenesis in HCV genotype 1b infected patients. While it was presumed that 70 Mutant strain could resist the PEG-IFN/RBV treatment, molecular genetic insights into the viral properties still remain limited.

Methods: Thirty four HCV 1b-infected patients who received a 48 week PEG-IFN/RBV treatment were enrolled. 1) 70 Wild/Mutant viral dynamics were evaluated by a 70 Wild/Mutant-specific real-time PCR. 2) Nucleotide sequences of full-length core of each patient were determined by cloning sequencing. 3) Relationships among 70 Mutant ratio, IL28B polymorphism, and treatment response were analyzed.

Results: 1) Before treatment, 25 (74%) patients were coinfected with 70 Wild/Mutant. 2) Co-existing 70 Mutant and 70 Wild did not show significant difference on the resistance toward PEG-IFN/RBV treatment while a higher 70 Mutant ratio was significantly associated with higher possibility of NVR ($P < 0.01$). 3) Interestingly, relapsers predominantly with 70 Wild at baseline showed 70 Mutant predominance at the early stage of relapse while later returning to 70 Wild predominance, Moreover, the predominant 70 Wild sequence after relapse was originated from 70 Wild sequence observed at baseline. 4) Univariate analyses showed that 70 Mutant ratio was associated with IL28B polymorphism ($P=0.04$) and platelet count at baseline ($P=0.014$). 5) Multivariate logistic regression showed 70 Mutant ratio was an independent predictor for NVR while IL28B was the strongest predictor for SVR.

Conclusions: The core 70 Mutant strain was rather a product selected by the complex interactions among virus and host immune system than that merely selected by the PEG-IFN/RBV treatment. Further understanding of these HCV variants 70 Wild/Mutant presented

in an infected host is likely to be of increasing importance to identify the most appropriate treatment for infected individuals. Novel therapeutic strategies targeting the 70 Mutant strain capable of replicating under low selection pressure may help to truly eliminate HCV infection without relapse.

INTRODUCTION

Approximately 170 million people had been chronically infected with hepatitis C virus (HCV) worldwide and almost half of them reside in Asia [44,45,46]. Similar as HBV, persistent HCV infection could induce a series of end-stage liver diseases including HCC. Each year more than 350,000 people die of the HCV-related liver diseases. HCV is a small (55-65 nm in size), enveloped, positive-sense single-stranded RNA belonging to the Hapacivirus genus within the Flaviviridae family. The HCV particles contain a positive polarity RNA genome with 5' and 3' UTRs and a long ORF which encoding a polyprotein precursor of around 3,000 amino acids. The polyprotein is then posttranslationally cleaved into at least ten mature proteins through host peptidase and viral protease activities [47]. The core protein, and two envelope proteins (E1, E2) are classified into structural protein, while NS2, NS3, NS4A, NS4B, NS5A, and NS5B are non-structural proteins.

Because of its error-prone replication and an overall high replication rate, HCV infection often involves genetically diverse but related groups of sequences or viral quasispecies [48]. Based on more than 30% nucleotide sequence differences, HCV could be divided into 6 genotypes and each genotype could be further split into multiple subtypes, a, b, c, etc. The global distribution of HCV genotypes is diverse while genotype 1b is the most

prevalent (most of North America, Northern and Western Europe, South America, Asia and Australia) (Figure 11).

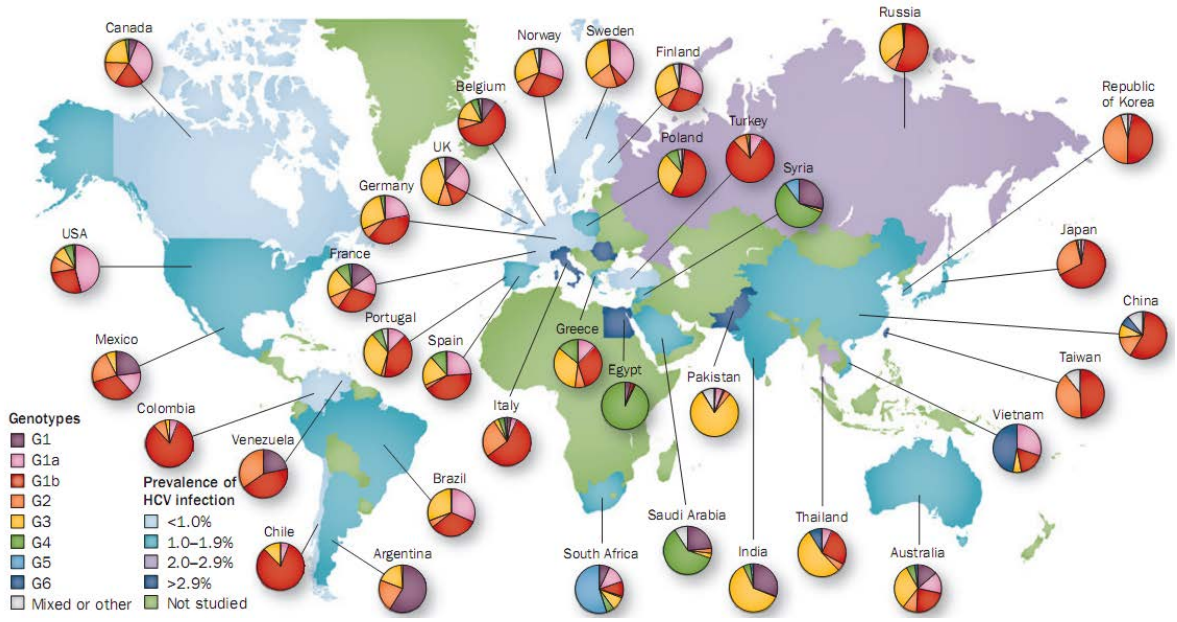


Figure 11. The global prevalence and geographic distribution of hepatitis C virus with different genotypes. The global distribution of HCV genotypes is diverse while genotype 1b is most prevalent (most of North America, Northern and Western Europe, South America, Asia and Australia). **(**Source: Hajarizadeh, B. et al. *Nat. Rev. Gastroenterol. Hepatol. 2013*)

Although advances in prevention/treatment options for this public health problem are rapidly emerging, no vaccine is available yet. Moreover, the efficacy of current standard of care (SOC), a combination therapy of Pegylated Interferon (PEG-IFN) and Ribavirin (RBV) is limited towards certain genotype/subtypes. For instance, compared with genotype 2 and 3, genotype 1b is more difficult to cure by PEG-IFN/RBV treatment and had a higher ratio of relapse. Only around 50% of naive patients infected by HCV genotype 1b could achieve sustained virologic response (SVR) while 70%-80% of genotype 2 or 3-infected patients did. Moreover, around 30% of genotype 1b-infected patients experience viral relapse after temporary viral clearance (Figure 12). The mechanism underlying the more resistance of

patients infected with HCV genotype 1b to PEG-IFN/RBV treatment remains elusive. Multiple host- and virus-related factors have been so far believed to participate in it.

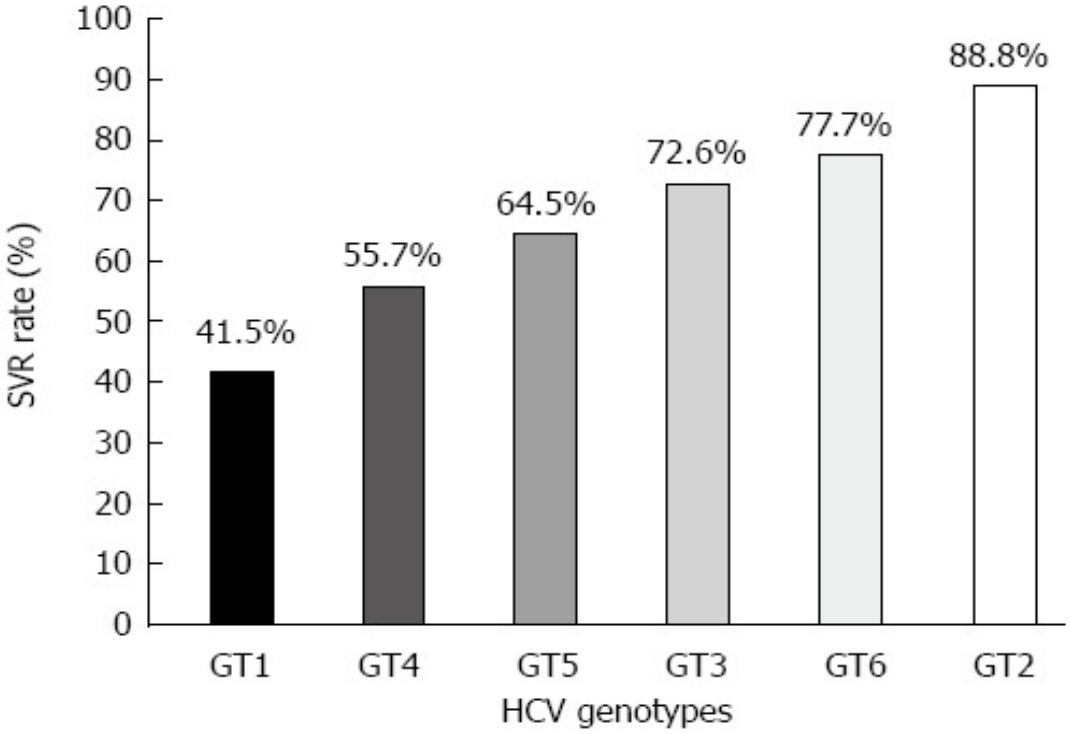
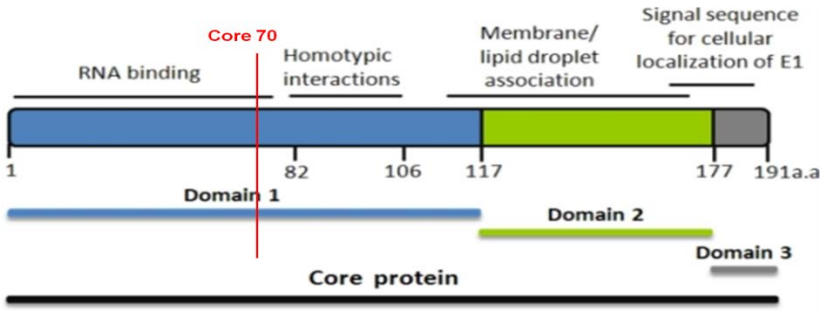


Figure 12. The averaged SVR rates toward PEG-IFN/RBV treatment among patients infected with different HCV genotypes. Patients infected with HCV genotype 1 presented the lowest SVR rate when compared with patients with other genotypes **(**Source: Ahmed El-Shamy et al. *World J Gastroenterol. 2014*).

Recently, a single amino acid substitution occurring in HCV core region amino acid (AA) 70 substitution [Arg/R (70 Wild) to Gln/Q (70 Mutant)] was reported to be significantly associated with treatment response towards PEG-IFN/RBV based treatment among HCV genotype 1b-infected patients [49,50,51,52,53,54] (Figure 13). Moreover, it was also revealed that the existence of core 70 Mutant before the treatment was associated with the development of HCC [55,56,57,58]. Even in patients who had achieved viral clearance, those with 70 Mutant strain infection would have a higher risk (Hazard ratio: 10.5) for HCC than those without [59]. All these data indicated the importance of 70 Mutant strain in disease

progression though molecular genetics insights into the viral properties still remain very limited.

A



B

	70	80	90	100	110	
Consensus	RRQPIPKARR	PEGRTWAQPG	YPWPLYGNEG	LGWAGWLLSP	RGSRPSWGPT	Efficac
HCJ	----------	----------	M---------	----------	----------	
Case 1	---------Q	----------	--L-------	M---------	----------	NVR
2	----------	----D-----	----------	M---------	----------	NVR
3	----------	----------	----------	M---------	---------N	NVR
4	----------	----------	----------	M---------	----------	NVR
5	----------	----------	----------	M---------	----------	NVR
6	---------Q	----A-----	----------	M---------	---------N	NVR
7	---------Q	----A-----	----------	----------	---------S	NVR
8	---------Q	----A-----	----------	----------	----------	NVR
9	---------Q	----P-----	----------	M---------	----------	NVR
10	---------Q	----A-----	----------	M---------	---------N	NVR
11	---------Q	----A-----	----------	M---------	----------	NVR
12	---------H	----A-----	----------	----------	----------	NVR
13	----------	----------	----------	M---------	----------	VR
14	----------	----A-----	----------	----------	-----N----	VR
15	----------	----------	----------	M---------	----------	VR
16	---------H	----D-----	----------	M---------	----------	VR
17	----------	----S-----	----------	----------	H---------	VR
18	----------	----A-----	----------	----------	----------	VR
19	----------	----------	----------	----------	----------	VR
20	----------	----------	----------	----------	H---------	VR
21	----------	----A-----	----------	---T------	----------	VR
22	----------	----A-----	----------	----------	---------S	VR
23	----------	----------	----------	----------	----------	VR
24	----------	----------	----------	----------	----------	VR
25	----------	----------	----------	----------	----------	VR
26	----------	----A-----	----------	----------	----------	VR
27	----------	----A-----	----------	----------	----------	VR
28	----------	----V-----	----------	M---------	-----N----	VR
29	---------Q	----A-----	----------	M---------	----------	VR
30	---------Q	----------	----------	----------	----------	VR
31	---------Q	----A-----	----------	M---------	----------	VR
32	---------H	----------	----------	M---------	----------	VR
33	----------	----------	----------	M---------	----------	VR
34	----------	----------	----------	M---------	---------N	VR
35	----------	----A-----	----------	----------	----------	VR
36	----------	----A-----	----------	----------	----------	VR
37	----------	----------	----------	----------	----------	VR
38	----------	----P-----	----------	----------	----------	VR
39	----------	----------	----------	M---------	----------	VR
40	---------Q	----A-----	----------	----------	-----N----	VR
41	----------	----------	----------	M---------	H----N----	VR
42	----------	----------	----------	----------	-----N---S	VR
43	----------	----------	----------	M---------	----------	VR
44	---------Q	----------	----------	M---------	----------	VR
45	----------	----------	----------	----------	----------	VR
46	----------	----A-----	----------	----------	---------N	VR
47	----------	----------	----------	----------	----------	VR
48	----------	----A-----	----------	----------	----------	VR
49	----------	----------	----------	M---------	---------A	VR
50	----------	----A-----	----------	----------	----------	VR

Figure 13. HCV genotype 1b infected patients presented different viral responses toward PEG-IFN/RBV treatment. (A) The location of core 70 in HCV core protein. (B) Sequences of amino acids 61–110 in the core region at the commencement of combination

therapy in 50 Japanese patients infected with HCV genotype 1b. Dashes indicated amino acids identical to the consensus sequence of genotype 1b, and substituted amino acids were shown by standard single-letter codes. Substitutions at amino acid 70 of the HCV core [Arg (R) to Gln (Q)] were significantly more frequent in NVRs (n=8, 66.7%) than VRs (n=7, 18.4%; P=0.003). NVR, null virological response; VR, virological response (Source: Akuta N. et al. *Intervirology. 2005).*

Host factors contributing to the low antiviral response among HCV genotype 1b infected patients were not well understood until 2009 when critical cases were reported. All of those papers were focused on IL28B, a host factor strongly related to HCV antiviral treatment responses [60,61,62]. They reported that patients carrying the favorable IL28B allele (rs12979860 CC or rs8099917 TT) would have a significantly better response toward PEG-IFN/RBV treatment than those carrying the unfavorable IL28B allele (rs12979860 CC/TT or rs8099917 GT/GG). Patients infected with genotype 2/3 usually carry favorable IL28B allele than patients infected with HCV genotype 1 [63]. Also Asians have a higher ratio of favorable IL28B allele than Caucasians and African-Americans [62]. Numerous studies have been performed consequently to further confirm the role of IL28B in HCV natural history and treatment while the mechanism of how IL28B affects host immune status and if there are crosstalks between IL28B and viral factors (i.e. Core 70 Mutant ratio) remain elusive.

In this study, we performed a core 70 Wild/Mutant-specific real-time PCR to examine the existence of HCV 70 Wild/Mutant strains among HCV genotype 1b-infected patients, and also we monitored the dynamic changes of core 70 Wild/Mutant in the process of antiviral treatment. Besides, 70 Wild/Mutant sequences from patients with different treatment responses/periods were analyzed, and we examined 70 Mutant ratio associated clinical and host factors in Japanese patients with HCV genotype 1b infection.

METHODS

Sera samples collection and preparation

Thirty-four HCV genotype 1b infected patients treated with PEG-IFN/RBV combination therapy at the Department of Gastroenterology in the University of Tokyo Hospital (Tokyo, Japan) during 2008-2010 were enrolled into analyses. Series of patients' sera were collected at different time points during treatment. At least three samples for each patient were collected. Sera were stored at -80 degrees before use.

HCV RNA extraction and reverse transcription

HCV RNA was extracted from the serum samples using QIAamp Viral RNA Mini Kit (QIAGEN) and cDNA was prepared by reverse transcription with random hexamers using Taqman Reverse Transcription Reagents (Applied Biosystems, ABI). The reaction conditions were 25℃ for 10 minutes and 42℃ for 40 minutes, and then 95℃ for 5 minutes, successively.

The control plasmids

The control plasmids used in our studies was pCXN2, an eukaryotic expression vector which was kindly provided by Dr. J. Miyazaki (Osaka University, Japan). Using the plasmid DNA controls as templates, wild type and mutant core genes were amplified with the following primers containing *Xho*I sites (underlined): forward primer, 5'-CCGCTCGAGACCATGAGC ACAAATCCTAAACCTCAA-3'; reverse primer, 5'-CCGCTCGAGTCAAGCGGAAGCTG

GGATGGT-3'. The amplified DNA fragments were digested with *Xho*I and then cloned into the *Xho*I site of pCXN2 to make the core protein expression plasmids: pCXN2-70 Wild (CGG) and pCXN2-70 Mutant (CAG). The plasmids were purified using High Pure Plasmid Isolation Kit (Roche) and identified by sequencing. Then the right clones were amplified and purified using HiSpeed Plasmid Maxi Kit (QIAGEN).

Core 70 Wild/Mutant specific real-time PCR

The HCV core 70 wild/mutant specific real-time PCR applied in our study was constructed by our ex-colleague Dr. Hu under the direction of Prof. Kato and Dr. Muroyama [64,65]. According to the variability of each position, the consensus sequence of HCV-1b CR and the sequences of plasmid DNA controls, the primers were found out from the conserved regions: forward primer, 5'-AGGAAGACTTCCGAGCGGTC-3'; reverse primer, 5'-CGGGGTGAC

AGGAGCCA-3'. The Taqman-MGB probes were designed to distinguish the HCV-70 Wild(CGG) and HCV-70 Mutant(CAG) strains as follows: 70 Wild, 5'-FAM-TCGCCGGCCCGAGG-MGB-3'; 70 Mutant, 5'-VIC-CTCGCCAGCCCGAGGG-MGB-3'. The real-time PCR was performed in a final volume of 50 μL containing 5 μL of the cDNA reaction, 0.6 μM of each primer, 0.1 μM of probe and 25 μL of 2×Taqman Universal PCR Master Mix (ABI). Two separate reaction systems were prepared to detect the HCV-70 Wild and HCV-70 Mutant respectively, but the two reactions were carried out in the same real-time PCR system at the same time. The cycle conditions were as follows: an initial denaturation for 10 minutes at 95 °C, followed by 45 cycles of denaturation for 15 seconds at 95 °C and annealing/extension for 1 minute at 60 °C. All the reactions were performed in triplicate using Stratagene Mx3000P Realtime PCR system.

Nucleotide sequencing of the core gene

We determined the sequences of the core gene both by cloning followed by sequencing. Briefly, nucleic acids were amplified by PCR using the following primers: forward primer, 5'-TAGCCGAGTAGTGTTGGGTC-3'; reverse primer, 5'-TTGGAGCAGTCGTTCGTG-3'. All samples were initially denatured at 95 °C for 10 minutes. The 40 cycles of amplification were set as follows: denaturation for 30 seconds at 95°C, annealing of primers for 30 seconds at 55°C, and extension for 1 minute at 72°C with additional 7 minutes for extension. The amplified PCR products were purified by the QIAquick PCR Purification Kit (QIAGEN, Tokyo) and then cloned into a plasmid (TOPO TA cloning: Invitrogen). At least 10 colonies were bidirectionally sequenced for each sera sample.

IL28B polymorphism determination

Human genomic DNA was extracted from peripheral blood using a blood DNA extraction kit (QIAGEN, Tokyo, Japan), according to the manufacturer's protocol. The allele typing of each DNA sample was performed by real-time PCR (Stratagene Mx3000P) using fluorescein-amidite-labeled SNP primer for the locus rs8099917 (Applied Biosystems).

Definition of treatment responses

According to the Asian Pacific Association for the Study of the Liver (APASL) guidelines for HCV, the sustained virological response (SVR) is defined as undetectable HCV RNA (<50 IU/mL) for 24 weeks after the end of therapy. Early virological response (EVR) refers to a >2 log reduction or complete absence of serum HCV RNA at week 12 of therapy compared with

the baseline level (cEVR). Null response (NVR) refers to less than 2 log decrease in HCV RNA (IU/mL) from baseline after 12 weeks of therapy. Partial response or partial non-response (PR) refers to more than 2 log10 decrease in HCV RNA (IU/mL) from baseline at 12 weeks of therapy, but detectable HCV RNA at week 24. Relapse refers to undetectable HCV RNA at the end of therapy, but reappearance of HCV RNA after the end of therapy (Figure 14). Nonresponder including two forms of virologic non-response: null response (NVR) and partial response (PR).

Statistical analysis

The data obtained were analyzed by Chi-square tests, paired-samples t tests and independent-samples t tests. A P value of <0.05 was considered statistically significant. Sequence analyses were performed by CLC sequence viewer 6 (CLC bio).

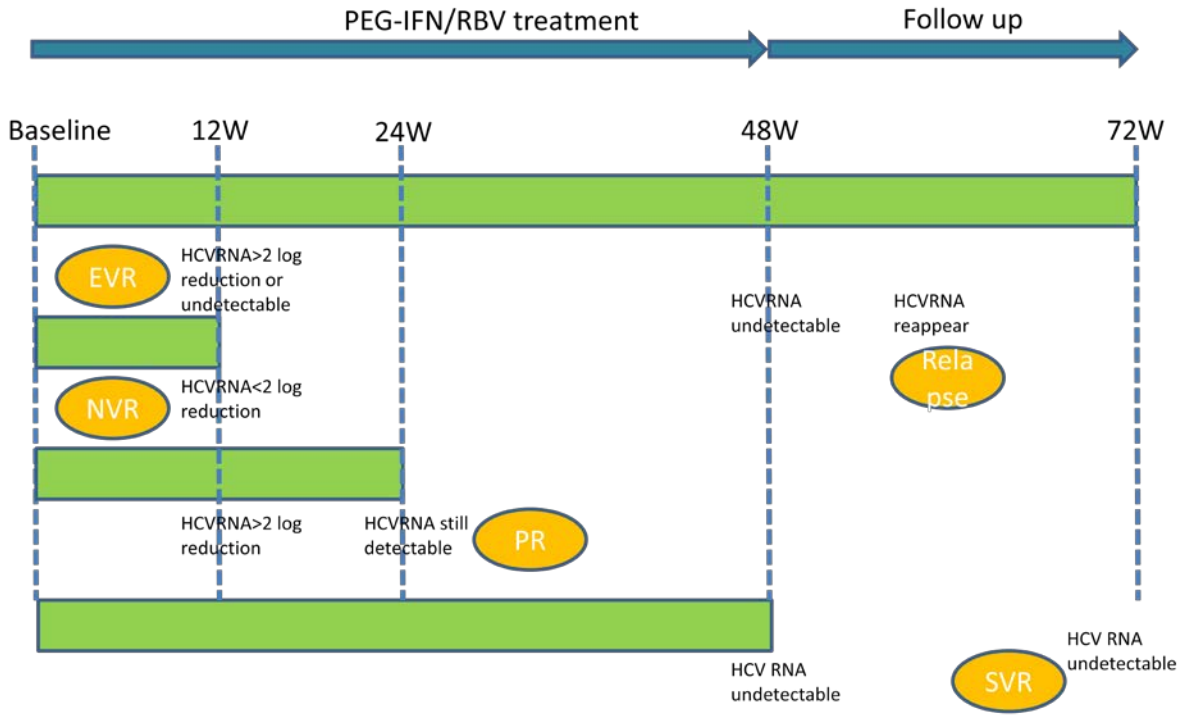


Figure 14. PEG-IFN/RBV treatment for enrolled patients infected with HCV genotype 1b and the definition of different treatment outcomes. Patients enrolled into this study accepted 48 weeks of PEG-IFN/RBV treatment succeeded by 24 weeks' follow-up. Different treatment outcomes were also shown. According to the APASL guidelines for HCV, SVR is defined as undetectable HCV RNA (<50 IU/mL) for 24 weeks after the end of therapy. EVR refers to a >2 log reduction or complete absence of serum HCV RNA at week 12 of therapy compared with the baseline level. NVR refers to less than 2 log decrease in HCV RNA (IU/mL) from baseline after 12 weeks of therapy. PR refers to more than 2 log10 decrease in HCV RNA (IU/mL) from baseline at 12 weeks of therapy, but detectable HCV RNA at week 24. Relapse refers to undetectable HCV RNA at the end of therapy, but reappearance of HCV RNA after the end of therapy.

Ethics statement

All patients enrolled into this study were originally from a multi-center, randomized study titled Inhibition of Hepatocarcinogenesis by Interferon Therapy (IHIT-II study). The IRB number registered in the University of Tokyo Hospital was P2007019-11X. This study protocol conformed to the ethical guidelines of the 2000 Declaration of Helsinki and the study was approved by the ethics committee of our institute with the IRB number 22-25.

RESULTS

Patient clinical characters at treatment baseline

Fourteen male and 20 female patients with HCV genotype 1b chronic infection were enrolled into analyses. The clinical features of the enrolled 34 patients were shown in Table 5 and Table 6. The average age of enrolled patients was 57.8 years old (range, 20-73 years) with a baseline average viral load at 5.8 log IU/ML.

Baseline viral quasispecies characters

Interestingly, we found most of enrolled patients (25/34, 74%) were co-infected by both core 70 Wild and 70 Mutant strains before treatment. The average mutant ratio of those 25 patients was 25.3%, with the lowest ratio at 0.48% and a highest ratio of 97.21% (Figure 15). In order to know if co-infection and mono-infection caused by different quasispecies, we compared 70 Wild and 70 Mutant sequences extracted from co-infection group (P9-P33) and mono-infection group (70 Wild, P1-8; 70 Mutant, P34), respectively. Phylogenetic trees did not show apparent discrepancies between co-infection group and mono infection group either for 70 Wild or 70 Mutant sequences (Figure 16). All sequences belonged to genotype 1b. Whole core sequence comparison showed one nucleotide position [(Nt541, CTG (co-infection 70 Wild) → TTG (mono-70 Wild infection)] had different nucleotide distribution (P=0.04) between co-infection 70 Wild and mono-infection 70 Wild sequences, but this mutation did not induce amino acid substitution, (Leu → Leu). Between co-infection 70 Mutant and mono-infection 70 Mutant, there were two sites with different nucleotide distribution but no amino acids were changed. [(Nt312, CGG (co-infection 70 Mutant) → CGA (mono-70 Mutant infection), and Nt507, T/CTG (co-infection 70 Mutant) → CTC (mono-70 Mutant infection)].

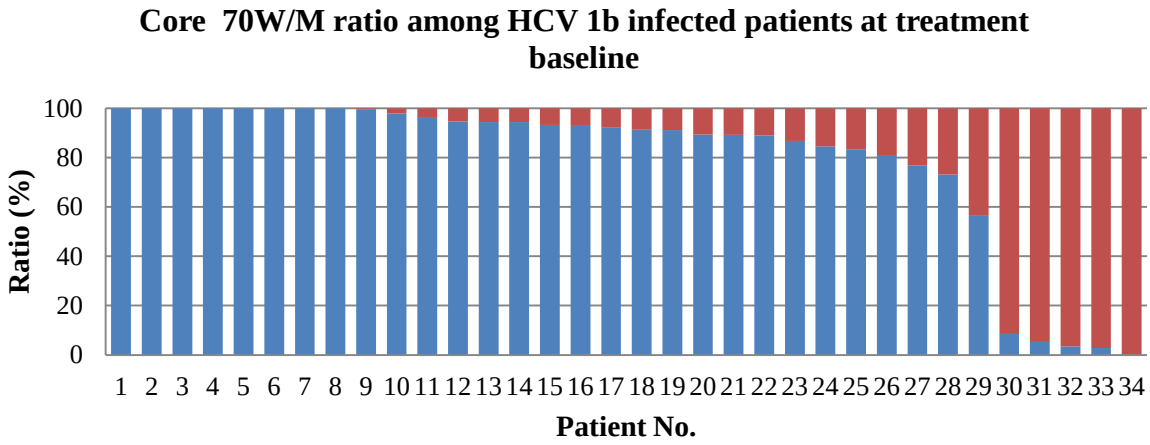


Figure 15. Core 70 Wild/Mutant ratio among HCV genotype 1b infected patients at the start of PEG-IFN/RBV treatment. We found that 25 out of 34 (74%) HCV 1b infected patients were co-infected by 70 Wild and 70 Mutant strains when PEG-IFN/RBV treatment started. (70 Mutant ratio: 0.48% - 97.21%). Eight patients were mono-infected by 70 Wild

and 1 patient had mono 70 Mutant infection. Blue column indicated the core 70 Wild ratio and the red column indicated core 70 Mutant ratio.

Table 5. Baseline characteristics of enrolled patients and treatment responses toward PEG-IFN/RBV treatment.

Variables	Mean (Range)
Age, in years	57.8 (20 - 73)
Gender (n/%)	
male	14 / 41.2%
female	20 / 58.8%
ALT. U/L	64 (18 - 225)
AST, U/L	80 (21 - 360)
GTP, U/L	57.3 (8.0 - 395.0)
PLT, $\times 10^4/mm^3$	14.0 (8.0-21.7)
Creatine, mg/dL	0.7 (0.5 - 0.9)
BMI	23.0 (17.7 - 30.9)
Tbil, mg/dL	0.8 (0.3 - 1.4)
IL28B genotype (n/%)	
major (TT)	26 / 76.5%
minor (CT/CC)	8 / 23.5%
HCVRNA level (log IU/ml)	5.8 (0.6 - 7.5)
NVR (n/%)	7 / 20.6%
EVR (n/%)	30 / 88.2%
SVR (n/%)	17 / 50%

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; PLT, platelets; BMI, body mass index; Tbil, total Bilirubin; NVR, null responder; EVR, early viral responder; SVR, sustained viral responder.

Table 6. Pretreatment HCVRNA level, core 70 Wild/Mutant ratios, and IL28B allele among patients with different treatment responses.

	Pretreatment HCVRNA (Mean) (IU/ml)	HCV Core 70 (wild: mutant)	IL28B (major: non-major)
SVR	4.31E+06	87.7% : 12.3%	16 : 1
NVR	8.99E+06	49.3% : 50.7%	1 : 6
Relapser	7.20E+06	90.1% : 9.9%	3 : 1
Nonresponder	1.03E+07	62.8% : 37.2%	7 : 6

Abbreviations: SVR, sustained viral responder; NVR, null responder; Nonresponder: including null responder (NVR) and partial responder (PR).

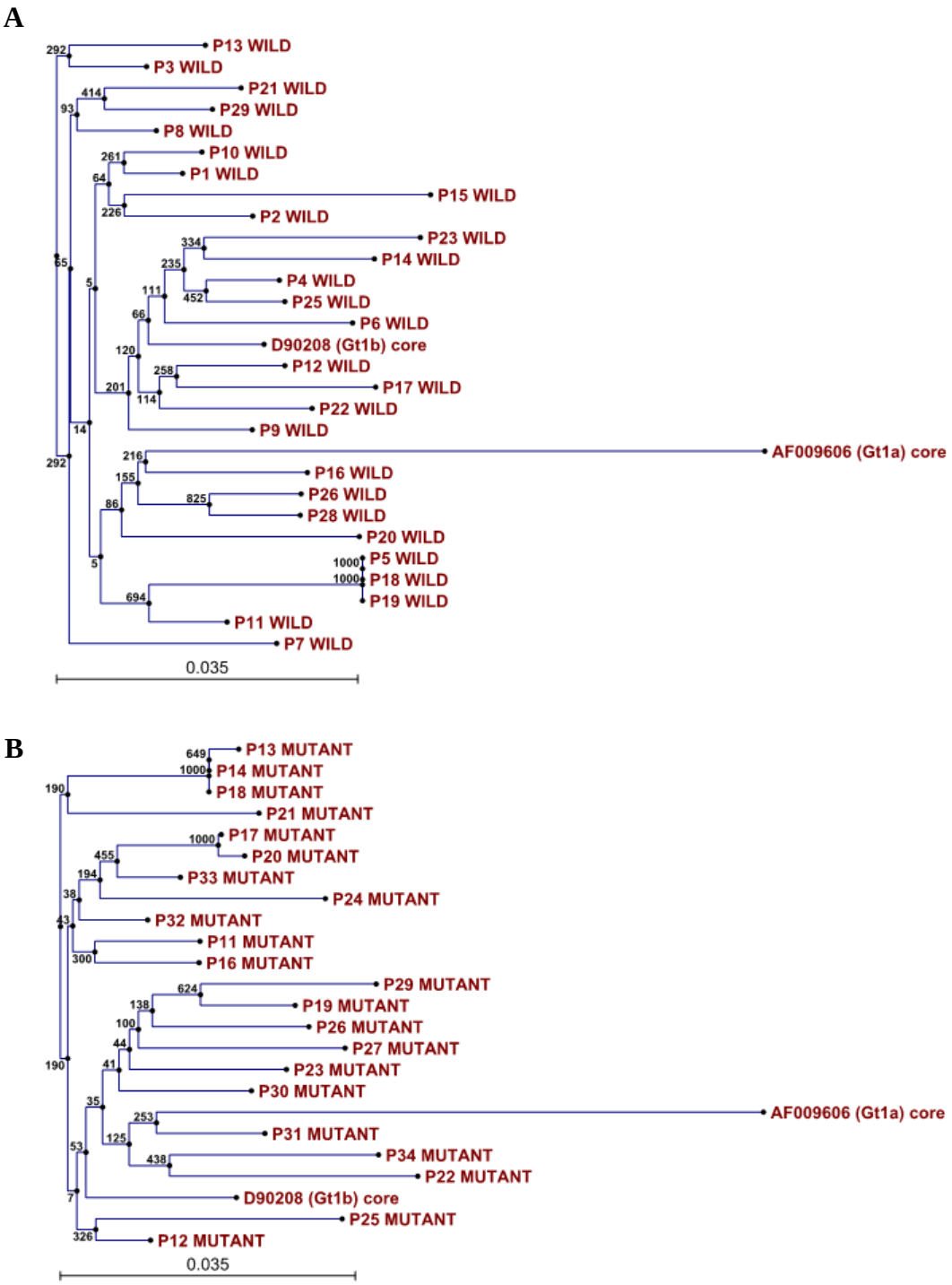


Figure 16. Phylogenetic tree for core 70 Wild/Mutant strains extracted from co-infection group and mono-infection group. Comparation of 70 Wild (A) and 70 Mutant (B) sequences extracted from co-infection group (P9-P33) and mono-infection group (70 Wild, P1-8; 70 Mutant, P34), respectively. Phylogenetic trees did not show apparent discrepancies between co-infection group and mono infection group either for 70 Wild or 70 Mutant sequences. All sequences belonged to genotype 1b. Two reference sequences (AF009606, genotype 1a; D90208, genotype 1b) downloaded from Refseq were used as outer control. (Refseq: http://www.ncbi.nlm.nih.gov/refseq/)

Patient treatment responses and baseline 70 Mutant ratio

In order to know if baseline 70 Mutant ratio plays roles in treatment responses, we compared the 70 Mutant ratio among groups with different treatment responses (Figure 17). In total 30 patients achieved EVR, including seventeen patients with cEVR. Seven patients had NVR and 17 patients achieved SVR. Four patients went through relapse after the end of therapy and 1 out of them relapsed at 16 weeks post-therapy, and the other 3 patients relapsed at 24 weeks post-therapy. The remaining 6 patients achieved PR. We found that NVRs showed an obviously higher pre-treatment 70 Mutant ratios than SVRs (P=0.013), while no any significant differences were found among other groups.

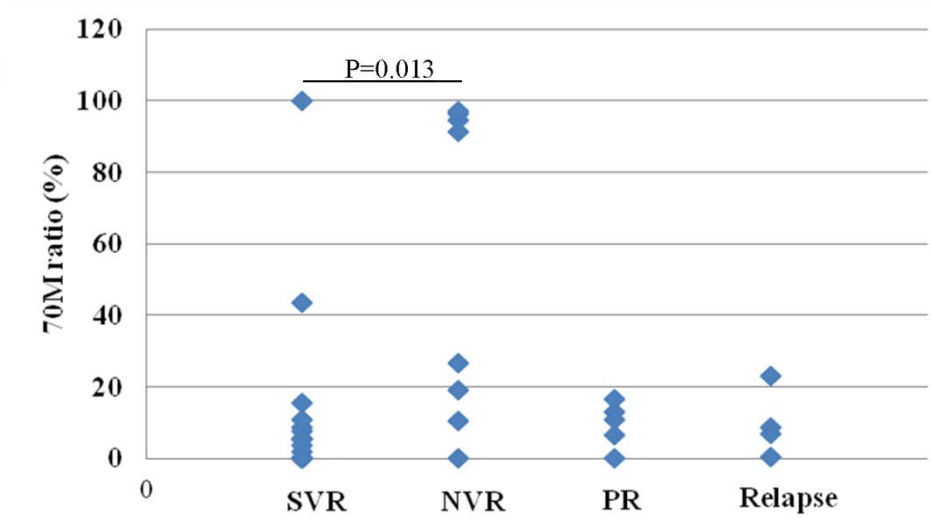
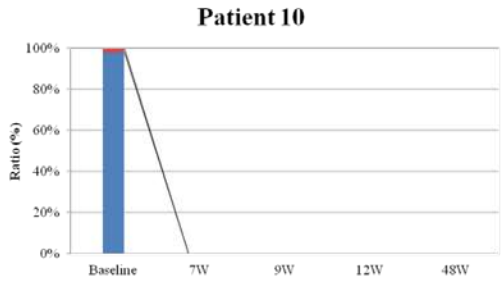


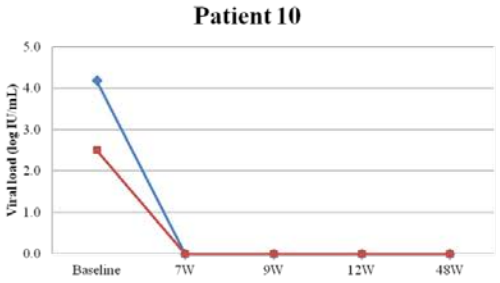
Figure 17. Baseline core 70 Mutant ratio among HCV 1b infected patients and treatment responses. NVRs showed a significantly higher pretreatment 70 Mutant ratio than SVRs (P=0.013).

The 70 Wild/Mutant dynamic changes during treatment

In order to know if 70 Mutant ratio changes during treatment, we next examined the dynamic changes of 70 Wild/Mutant among co-infection patients. With respect to SVR, which in total 10 out of 25 co-infected patients achieved (P10-14, P17, P19, P22, P24, and P29), the 70

Wild/Mutant dynamic changes during treatment among them were shown in Figure 18. Very interestingly, viral load decreased with the procession of Peg-IFN/RBV treatment, while the 70 Mutant ratio kept no significant changes in each patient when compared with baseline level ($P>0.05$). In NVR, 6 (P20, P26, P28, P31-33) out of 7 NVRs were co-infected with 70 Wild/Mutant before treatment. From sera sample available, we found that the mode of 70 Wild/Mutant response to PEG-IFN/RBV treatment was similar to those of SVRs, that is, the co-existing 70 Mutant was not shown to be more resistant than 70 Wild, including patients with very high ratio of 70 Mutant (P28, P31-33) (Figure 19). We compared the pre-treatment 70 Wild sequences obtained from SVRs and NVRs who were co-infected by both quasispecies. Four C/T transitions were found between the two groups [(Nt378, CTC (SVR) → CTT (NVR), Nt465, GTC (SVR) → GTT (NVR), Nt534, CTC (SVR) → CTT (NVR), and Nt561, ATC (SVR) → ATT (NVR)], none of which induced nonsynonymous substitution. Whether these alterations in 70 Mutant sequence could help the quasispecies to keep a predominant amount in host need further analyses. With regard to 70 Mutant from SVRs and NVRs, we didn't find any significant difference between two groups. As for relapse, 4 out of 34 patients experienced relapse during follow-up and all of them were co-infected before treatment. In a patient relapser 3, viruses were found to reappear at 16 weeks post-treatment and in the other 3 relapsers that was at 24 weeks post-treatment. We did not find a higher baseline 70 Mutant ratio among these 4 relapsers than SVRs ($P>0.05$). However, interesting viral dynamic changes were observed from these relapsers (Figure 20): 1) the 70 Mutant strain was presented at the early period of relapse at 75% (3/4) relapsers, even as predominant type. 2) In later period after relapse, 70 Wild turned into the predominant strain. 3) Sequences analyses revealed that the predominant 70 Wild sequences after relapse were originated from 70 Wild sequences observed at treatment baseline.


Patient 10
100%
80%
60%
40%
20%
0%
Ratio (%)
Baseline
7W
9W
12W
48W


Patient 10
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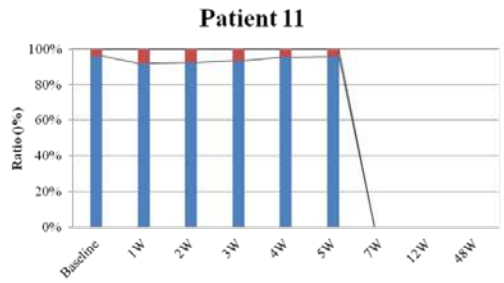
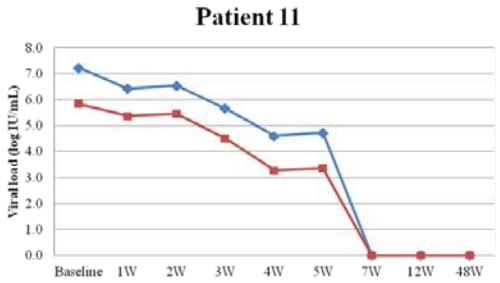
4.0
3.0
2.0
1.0
0.0
Viral load (log IU/mL)
Baseline
7W
9W
12W
48W


Patient 11
100%
80%
60%
40%
20%
0%
Ratio (%)
Baseline
1W
2W
3W
4W
5W
7W
12W
48W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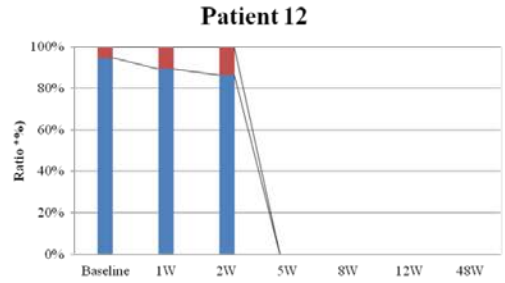
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Viral load (log IU/mL)
Baseline
1W
2W
3W
4W
5W
7W
12W
48W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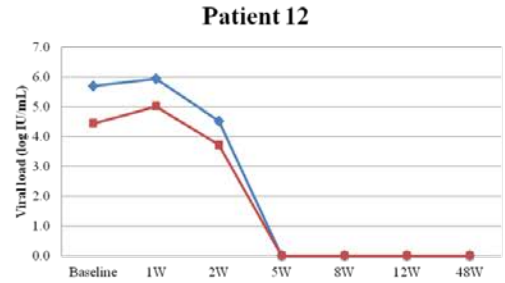
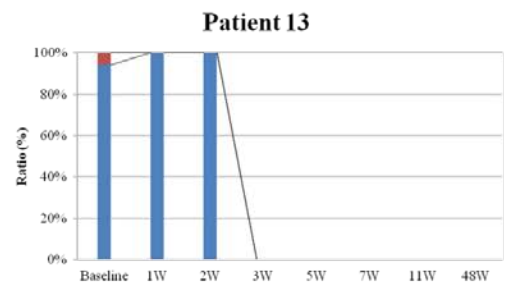
80%
60%
40%
20%
0%
Ratio (%)
Baseline
1W
2W
5W
8W
12W
48W


Patient 12
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Viral load (log IU/mL)
Baseline
1W
2W
5W
8W
12W
48W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100%
80%
60%
40%
20%
0%
Ratio (%)
Baseline
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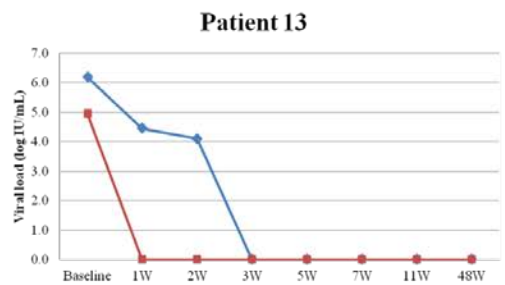
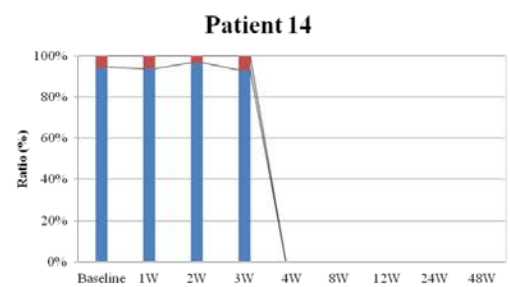
2W
3W
5W
7W
11W
48W


Patient 13
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Viral load (log IU/mL)
Baseline
1W
2W
3W
5W
7W
11W
48W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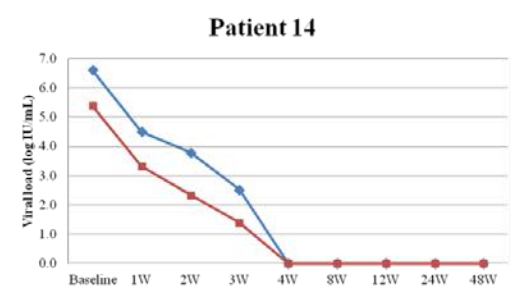
80%
60%
40%
20%
0%
Ratio (%)
Baseline
1W
2W
3W
4W
8W
12W
24W
48W


Patient 14
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Viral load (log IU/mL)
Baseline
1W
2W
3W
4W
8W
12W
24W
48W

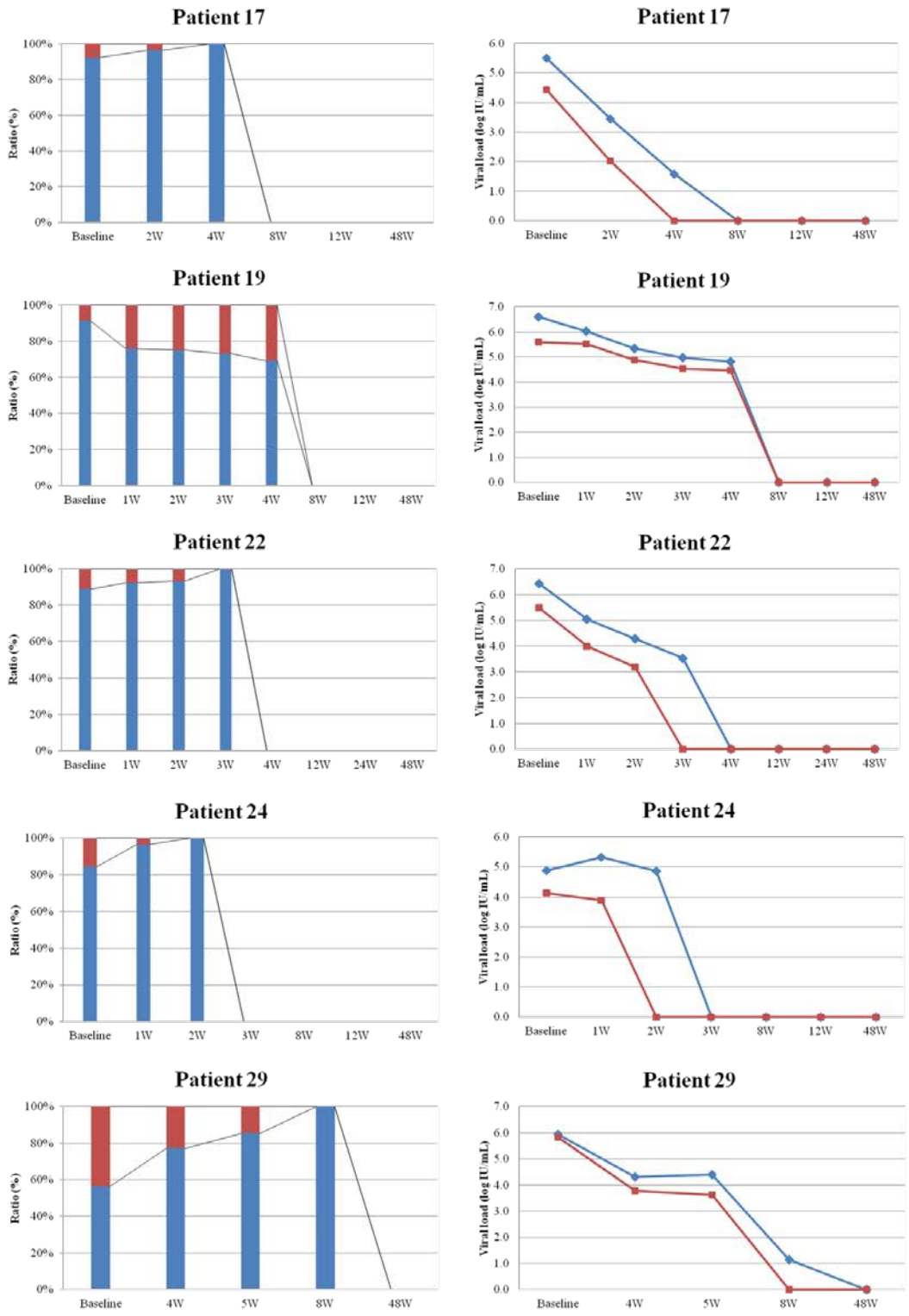
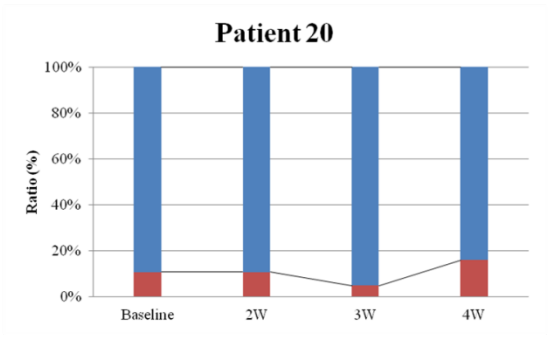


Figure 18. Core 70 Wild/Mutant dynamic changes during PEG-IFN/RBV treatment among SVRs. Graphs on the left showed the dynamic changes of 70 Wild/Mutant ratio on different timepoints (Red: 70 Mutant ratio, Blue: 70 Wild ratio). Graphs on the right showed the dynamic changes of 70 Wild/Mutant viral loads on different time points (Red: 70 Mutant viral load, Blue: 70 Wild viral load). Among SVRs, the ratio of 70 Wild/Mutant didn't change significantly during treatment. All patients shown in this figure carried major type of IL28B allele.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Ratio (%)
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80%
60%
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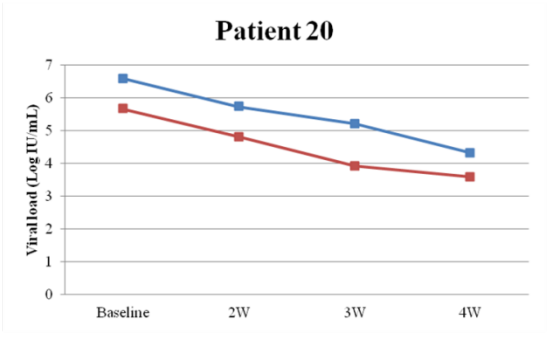
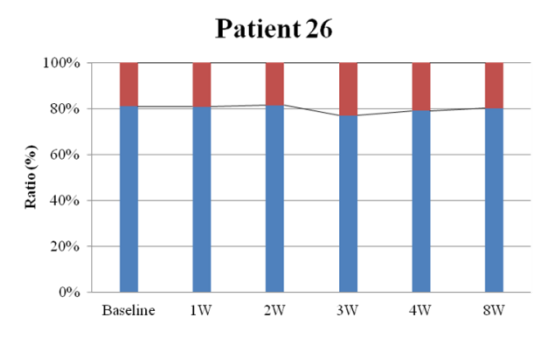
20%
0%
Baseline
2W
3W
4W


Patient 20
Viralload (Log IU/mL)
7
6
5
4
3
2
1
0
Baseline
2W
3W
4W


Patient 26
Ratio (%)
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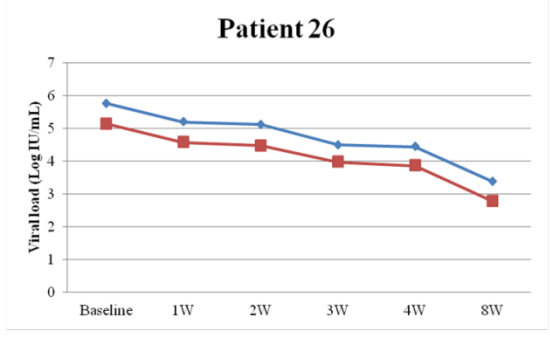
80%
60%
40%
20%
0%
Baseline
1W
2W
3W
4W
8W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Viralload (Log IU/mL)
7
6
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4
3
2
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Baseline
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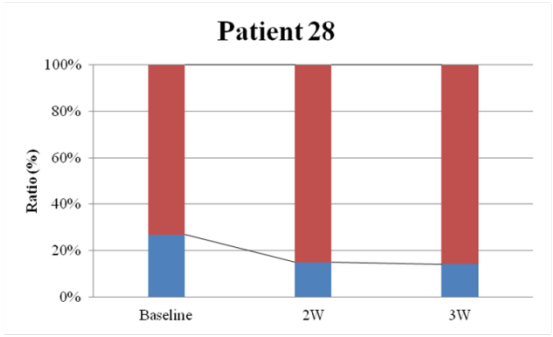
2W
3W
4W
8W


Patient 28
Ratio (%)
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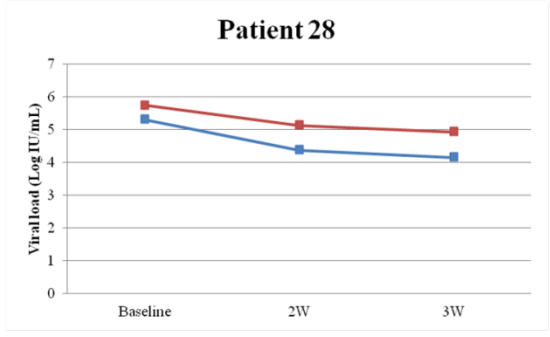
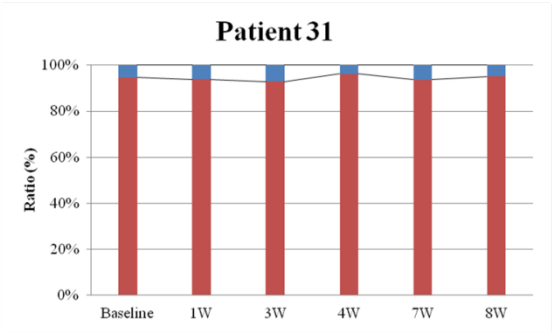
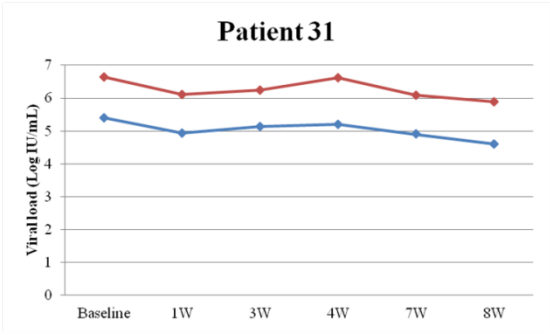
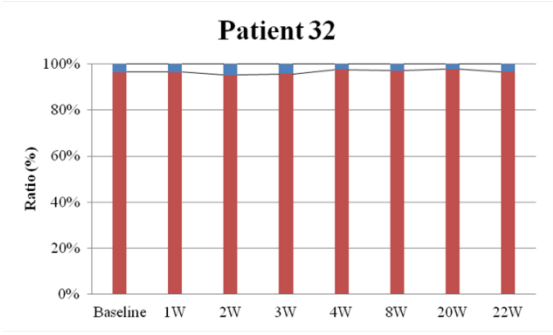
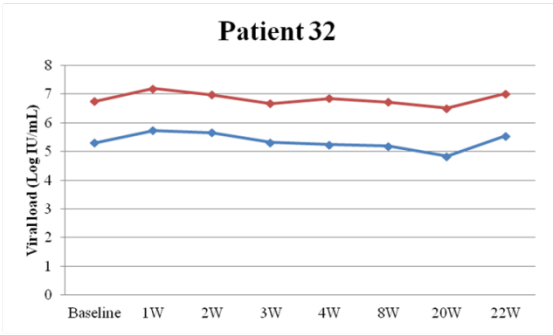
60%
40%
20%
0%
Baseline
2W
3W


Patient 28
Viralload (Log IU/mL)
7
6
5
4
3
2
1
0
Baseline
2W
3W


Patient 31
Ratio (%)
100%
80%
60%
40%
20%
0%
Baseline
1W
3W
4W
7W
8W


Patient 31
Viralload (Log IU/mL)
7
6
5
4
3
2
1
0
Baseline
1W
3W
4W
7W
8W


Patient 32
Ratio (%)
100%
80%
60%
40%
20%
0%
Baseline
1W
2W
3W
4W
8W
20W
22W


Patient 32
Viralload (Log IU/mL)
8
7
6
5
4
3
2
1
0
Baseline
1W
2W
3W
4W
8W
20W
22W

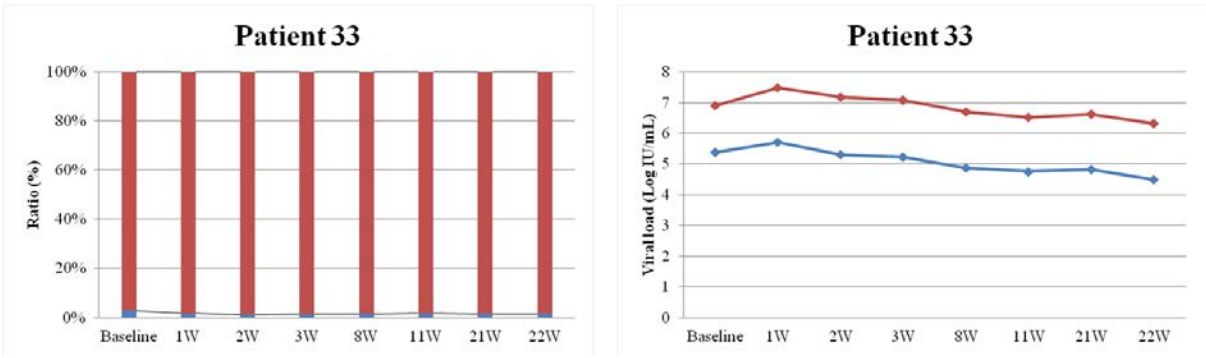


Figure 19. Core 70 Wild/Mutant dynamic changes during PEG-IFN/RBV treatment among NVRs. Graphs on the left showed the dynamic changes of 70 Wild/Mutant ratio on different time points (Red: 70 Mutant ratio, Blue: 70 Wild ratio). Graphs on the right showed the dynamic changes of 70 Wild/Mutant viral loads on different time points (Red: 70 Mutant viral load, Blue: 70 Wild viral load). the mode of 70 Wild/Mutant responded to PEG-IFN/RBV treatment was similar as in SVRs, that is, the co-existing 70 Mutant didn't show to be more resistance than 70 Wild, including patients with very high ratio of 70 Mutant (P28, P31-33). IL28B allele: Patient 20, major type; Patient 26, 28, and 31-33, non-major type.

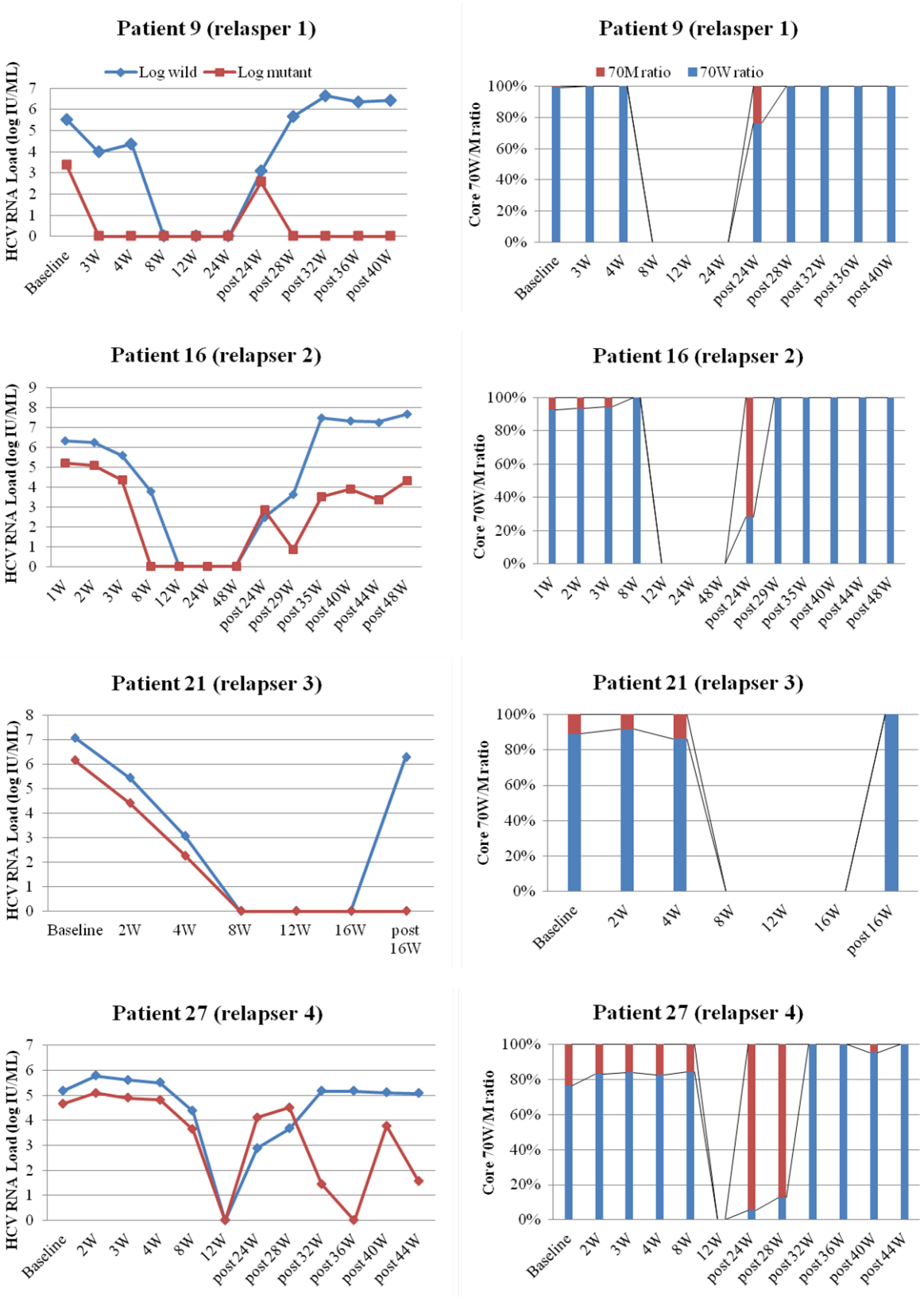


Figure 20. The core 70 Wild/Mutant dynamic changes during PEG-IFN/RBV treatment among four relapsers. Line in blue indicated 70 Wild and red line referred to 70 Mutant. Graphs on the left showed the viral load changes during treatment and Graphs on the right

showed the 70 Wild/Mutant ratio during treatment. IL28B allele: Patient 9, 16 and 21, major type; Patient 27, non-major type.

Baseline 70 Mutant ratio as predictor for final treatment responses

We wondered if baseline 70 Mutant ratio could be used as a predictor for antiviral treatment, and therefore the association among factors including 70 Mutant ratio and treatment responses were examined by Uni- and Multi-variates analyses. Univariate analysis showed that lower core 70 Mutant ratio was easier to achieve SVR (P=0.02), and non-NVR (P<0.01) (Mann-Whitney U test). Multivariate analysis revealed that baseline 70 Mutant ratio was an independent risk factor for NVR but IL28B was the strongest determinant for final outcomes (Table 7). Moreover, core 70 Mutant ratio was found to be associated with two host factors, IL28B polymorphism (P=0.04) and platelet count (P=0.014) at baseline (Table 8).

Table 7. Uni- and multivariate analyses for final treatment responses.

Variable	SVR				NVR			
	Univariate	Multivariate			Univariate	Multivariate		
	P value	OR	95% CI	P value	P value	OR	95% CI	p value
Age	0.86				0.78			
Gender	0.49				0.17			
TBiL	0.15				0.24			
ALT	0.22				0.39			
AST	0.38				0.57			
GTP	0.01	1.04	(0.99-1.10)	0.08	0.06			
Creatine	0.93				0.57			
PLT	0.15				0.04	1.13	(0.73-1.75)	0.59
HCV RNA	0.01	1	(1.00-1.00)	0.07	0.38			
Body weight	0.25				0.89			
BMI	0.58				0.98			
Core 70 Mutant ratio	0.02	1.02	(0.99-1.05)	0.31	<0.01	0.97	(0.93-1.00)	0.04
IL28B	0.02	17.11	(1.28-229.46)	0.03	<0.001	0.05	(0.01-0.72)	0.02
NS5A ISDR	0.98				0.89			

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; PLT, platelets; BMI, body mass index; Tbil, total Bilirubin; NVR, null viral response; SVR, sustained viral response.

Table 8. Core 70 Mutant related clinical factors.

Variables	Core 70 wild	Core 70 mutant	Univariate analysis* (P value)	Multivariate analysis (P value)
Age, in years	57.38	60.00	0.79	
Gender (n/%)			0.36	
male	11/37.9%	3/60%		
female	18/62.1%	2/40%		
ALT. U/L	63.66	68.80	0.54	
AST, U/L	78.69	92.40	0.37	
GTP, U/L	58.10	52.60	0.33	
PLT, $\times 10^4/mm^3$	14.54	10.76	0.01	0.04
Creatine, mg/dL	0.68	0.64	0.45	
Tbil, mg/dL	0.84	0.76	0.59	
IL28B genotype (n/%)			0.04	0.23
major (TT)	24/82.8%	2/40%		
minor (CT/CC)	5/17.2%	3/60%		
HCVRNA level, log IU/ml	6.74E+06	8.07E+06	0.9	

*Mann-Whitney Test

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; PLT, platelets; BMI, body mass index; Tbil, total bilirubin.

DISCUSSION

In many studies, core 70 Mutant has been considered as a PEG-IFN/RBV-resistant mutation, frequently reported to be a valuable biomarker for antiviral treatment responses and hepatocarcinogenesis. The traditional way of detecting this point mutation is direct sequencing, a qualitative analysis. While when patients were mixed infected by both core 70 Wild and Mutant strains, it'll be impossible to distinguish how many viruses had mutated only via direct sequencing. Moreover, little will be known about its actual response to

antiviral treatment *in vivo*. Through detailed virological characterization using a core 70 Wild/Mutant-specific real-time PCR system and cloning sequencing, we provided a new insight into viral dynamics during treatment in HCV genotype 1b-infected patients.

The prevalence of core 70 Wild/Mutant strain co-infection in HCV-infected subjects has been reported in up to 60% of those infected. This core 70 Mutant strain appeared more frequently in HCV genotype 1b than other genotypes, genotype 2a and 2b especially. Among our enrolled patients, most of them (74%) presented a co-infection of core 70 Wild/Mutant at the beginning of treatment. This may be because of the fact that our patients were chronically infected and some of them were not naive to treatment. While several studies based on healthy blood donors reported that around 40% blood donors who recently acquired HCV genotype 1b infection and are treatment-naive had presented this 70 Mutant strain. Moreover, this 70 Mutant was observed only in HCV genotype 1b-infected donors but not in patients infected with other genotypes such as genotype 2a and 2b [66]. These reflected that possibly 70 Mutant was not a treatment-selected subtype, at least not selected within a short period.

We next monitored the 70 Wild/Mutant dynamic changes during the treatment. We found that under the co-existing circumstance, both viral quasispecies (70 Wild/Mutant) showed similar resistance towards treatment. If the patients had responses to the treatment, the viral loads of mutant decreased as much as those of wild type. But if the patients had no responses, both the wild type and mutant resisted the treatments. Mixed HCV infection has been previously reported as competition between variants with the fitter strain having an advantage over others [67]. Similar phenomenon was observed from our patients, with the two viral quasispecies not propagated equally. Either 70 Wild or 70 Mutant should be a major quasispecies in host. We also found that the viral dynamic of all NVRs, failing to achieve viral cleanrance changes actually varied according to different basement core 70 Mutant ratios. NVRs with a relatively lower core 70 Mutant ratio tended to have viral decrease

during the treatment more or less as in Patient 20, 26, while the virus level of NVRs with a higher core 70 Mutant ratio did not decrease during the treatment as in Patient 31-33. In addition, quasispecies from co-infection host or mono-infection host were not significantly different in sequences.

A few previous studies had reported that core 70 Mutant was associated with poorer response. We therefore wondered if there are certain association between core 70 Mutant ratio and treatment responses and if this factor could be applied to clinical practice. We divided the enrolled 34 patients into four groups by their treatment responses (Table 6). Very interestingly, NVRs possessed the highest HCV core 70 Mutant ratio (50.7%) when compared to SVR, Relapser, and Nonresponders (12.3%, 9.9%, and 37.2%, respectively). Uni- and multivariate analyses revealed that 70 Mutant ratio was a risk factor for NVR. The result emphasized the potential diagnostic and prognostic values of this quantitative analysis in clinical practice.

Recently the association of IL28B gene polymorphism and treatment responses have been extensively investigated [60,61,68]. This single nucleotide polymorphism (SNP) also showed significant correlation with natural HCV clearance, indicating that this host factor may participate in innate immune processes [62]. We therefore examined the association between core 70 Mutant ratio and clinical/host factors including IL28B. Very intriguingly, the core 70 Mutant ratio was related to IL28B polymorphism and platelet count before treatment by univariate analysis. While in multivariate analysis, only baseline platelet count revealed to be associated with core 70 Mutant ratio. In patients with chronic liver diseases, a decreased platelet count usually indicates the damage of liver function and fibrosis progression. Therefore, core 70 Mutant ratio is possibly associated with advanced liver diseases though it is hard to say which is the cause and effect. Our result was consistent with study from Dr. Kobayashi et al [69].

Relapse remains to be one of the difficult problems for CHC treatment especially in HCV genotype 1b-infected patients and the molecular mechanism remains unclear. In our enrolled patients, 4 of them experienced viral relapse at 24W post-therapy or even earlier. Whether those relapsed viruses were derived from outside by reinfection and what kind of strain could survive under a low population and finally relapse remain to be clarified. By analyzing the viral sequences before and after relapse, we found that three of them initially showed a very low level of 70 Mutant, which became predominant later turning into 70 Wild as the predominant strain. This indicated that 70 Wild/Mutant could get adapted to different selective pressures. 70 Mutant strain may be easier to replicate under a low pressure and escape from host immune system while 70 Wild may have a higher replication ability possibly adapted to a higher selective pressure. As we mentioned in Introduction section, previous publications revealed that the existence of core 70 Mutant before the treatment was associated with the development of HCC [55,56,57,58]. Even in patients who had achieved viral clearance, those with 70 Mutant strain infection had a higher risk (Hazard ratio: 10.5) for HCC than those without [59]. Their results actually supported our finding that mutated core 70 strain may acquire a better “fitness”, or even “stemness” during that process though it has a relatively poorer replication capacity. In addition, the predominant 70 Wild sequences after relapse didn't present significant nucleotide changes compared with 70 Wild sequence at baseline. This may partially explain that same regimen of Peg-IFN/RBV are still effective for many patients after relapse if they showed virological responses towards PEG-IFN/RBV treatment at the very beginning.

Another noteworthy point in this study is the sequence variance among quasispecies from groups with different treatment responses and time points. It is well known that HCV core protein could participate in multiple cellular functions including apoptosis, oncogenic signaling, lipid metabolism, and transcriptional activation [70,71] and demonstrated

oncogenic potential in transgenic mice [5]. Therefore sequence changes in core sequence may have altered those functions, and further investigation is warranted for novel mechanisms of action.

Overall, the core 70 Mutant strain was rather a product selected by the complex interactions among virus and host immune system than that merely selected by the PEG-IFN/RBV treatment. A further understanding of these HCV variants 70 Wild/Mutant presented in an infected host is likely to be of increasing importance in order to identify the most appropriate treatment for infected individuals. Novel therapeutic strategies targeting the 70 Mutant strain capable of replicating under low selection pressure may help to truly eliminate HCV infection without relapse.

CLOSING REMARKS

In recent years, increasing attention has been given to HBx mutations that potentially governing HBV related carcinogenesis. Only a few studies revealed certain mutations were risk factors for HCC such as BCP mutations. However, those studies were mainly based on patient data from local areas and the number of recruited patients is usually limited. Therefore, contradictory results inevitably exist. To until now, little is known about whether the impacts of those identified mutations are true also in other areas and genotypes.

The development of large-scale public virus database enabled us to investigate the HBV viral character in an overall perspective. In this study, based upon a global HBV sequence database, we compared HBx sequences between genotype C-infected HCC and Non-HCC patients originated from different countries. Sixteen nucleotide differences between the two groups were found and 7 of them (A1383C, R1479Y, C1485T, C1631T, C1653T, G1719T, and T1800C) were further identified to be critical for genotype C HBV-related HCC. Three of them (R1479Y, C1631T and T1800C) were novel mutations identified in this study. Moreover, some of these mutations also pre-existed in other HBV genotypes, even as major types. But 1383C, 1479C, 1653T and 1719T seemed to be genotype C unique.

According to previous publications, mainly direct sequencing was used to investigate HCV core 70 Mutant. However, this method could only detect core 70 Mutant in a qualitative way. Nothing was known about the quantitative characters of virus. In our HCV study, we performed a quantitative analysis of HCV core 70 Wild/Mutant strains among patients infected with HCV genotype 1b during the PEG-IFN/RBV treatment. By monitoring the dynamic changes of core 70 Wild/Mutant during treatment, several interesting results were obtained.

The majority of our enrolled HCV genotype1b patients were mixed-infected by core 70 Wild/Mutant strains. A poor treatment response was associated with high basal core 70 Mutant ratio. NVRs possessed an average core 70 Mutant ratio at around 50% while SVRs also had approximate 15% Core 70 Mutant ratio. Such quantitative difference of virus could not be detected by direct sequencing. Data from relapsers revealed that Core 70 Mutant strain probably obtained the “fitness” or even “stemness” at the cost of replication capacity more or less. Hence the persistence of HCV core 70 Mutant strain in patients is highly likely to be the key for viral relapse and further carcinogenesis. To our knowledge, there had been no published studies addressing this point. Although still a few problems remain, we believe that the identification and analyses of the specific mutations of HCV core protein could provide clearer virological understanding of HCV with diagnostic and prognostic values that contributing to the personalized therapy among hepatitis viruses infected patients.

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