serum HBV DNA during immunosuppressive therapy and administer NAs should it be detected. The second measure is to administer NAs from the onset of immunosuppressive therapy. The third measure is to maintain circulating HBsAb titer using HB vaccines and/or HB immunoglobulins. Reports have suggested that regular evaluation of HBV DNA is effective in avoiding de novo hepatitis in patients treated with TNF-α inhibitors because HBV reactivation could be controlled by NAs when found at an early stage.[46,49] It is still unclear how often and for how long patients should be tested to detect HBV viremia. Prophylactic administration of NAs is also an option to preempt de novo hepatitis B due to TNF-α inhibitors because NAs are normally used to prevent reactivation in carrier patients. However, the issue of cost-efficiency versus relatively low incidence of de novo hepatitis B needs to be reconciled. Lastly, maintenance of circulating HBsAb titer using HB vaccines may be effective in responders since several studies[44,46] have shown that HBsAb titer decreases during TNF-α inhibitor therapy. As with HBV DNA monitoring and prophylactic NA administration, further studies are required to clarify the extent of HB vaccination effectiveness in preventing de novo hepatitis B due to TNF-α inhibitors.

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Novel Evidence of HBV Recombination in Family Cluster Infections in Western China

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Abstract

Two hepatitis B virus (HBV) C/D recombinants were isolated from western China. No direct evidence indicates that these new viruses arose as a result of recombination between genotype C and D or a result of convergence. In this study, we search for evidence of intra-individual recombination in the family cluster cases with co-circulation of genotype C, D and C/D recombinants. We studied 68 individuals from 15 families with HBV infections in 2006, identified individuals with mixed HBV genotype co-infections by restriction fragment length polymorphism and proceeded with cloning and DNA sequencing. Recombination signals were detected by RDP3 software and confirmed by split phylogenetic trees. Families with mixed HBV genotype co-infections were resampled in 2007. Three of 15 families had individuals with different HBV genotype co-infections in 2006. One individual (Y2) had a triple infection of HBV genotype C, D and C/D recombinant in 2006, but only genotype D in 2007. Further clonal analysis of this patient indicated that the C/D recombinant was not identical to previously isolated CD1 or CD2, but many novel recombinants with C2, D1 and CD1 were simultaneously found. All parental strains could recombine with each other to form new recombinant in this patient. This indicates that the detectable mixed infection and recombination have a limited time window. Also, as the recombinant nature of HBV precludes the possibility of a simple phylogenetic taxonomy, a new standard may be required for classifying HBV sequences.

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Introduction

Not all viruses are equally prone to recombination. Recombination has not been detected in several viruses despite repeated searches [1]. Whether recombination does or does not exist is important for understanding the evolution and replication mechanism of a specific kind of virus. Hepatitis B virus (HBV), a major human pathogen, has been classified into 10 genotypes and several sub-genotypes [2,3]. Many sub-genotypes were identified by polygenetic analysis as recombinants. But there is no direct evidence to indicate that these subgenotypes arose as a result of recombination or perhaps a result of convergence.

Coinfection with different HBV genotype strains is a prerequisite for recombination. As more than one genotype is predominant in most of the geographic regions, coinfection between the predominating HBV genotypes is not a rare finding, especially for B and C, or A and D. The prevalence of mixed HBV genotype infections has been reported using varied genotyping methods [4,5,6].

Our previous study found two kinds of HBV C/D recombinants in northwest China [7]. In a further study of ethnic groups of five provinces, we confirmed the geographic and ethnic distribution of the HBV C/D recombinant in northwest China [8], and found that family-cluster HBV infections were common in these endemic areas. We hypothesize that infected members of HBV family clusters would gain exposure to various genotypes through marriage, while at the same time; competent strains would be selected through vertical transmission. It would be useful to observe the mixed infection in family-cluster cases, especially in patients infected with C/D recombinants.

The aim of this study was to evaluate the possibility of recombination between two HBV genotypes within an individual by finding cluster-infected families in which individual members were infected with different HBV genotypes. We would then look for individuals within these families with multiple-genotypes that were likely to have been obtained from other family members as a result of vertical or horizontal transmission. Novel viral genomes within an individual with a multiple genotype infection that were mosaics of the known viral genotypes in the family, but not present in any of the other family members, would be consistent with the hypothesis that they arose within the individual with multiple genotype infections.

Methods

Subjects

We enrolled 68 patients with a chronic HBV infection from 15 families. All the families were from a district located at the boundary of Gansu and Qinghai provinces, where the prevalence of genotype C2, D1 and C/D recombinant HBV were known to be high [8]. The families were initially identified with cluster HBV infection in an epidemiological survey in 2002. Sixty-eight individuals were sampled in June 2006 and December 2007 for the purpose of assigning HBV genotypes to chronically infected individuals and finding individuals with multiple HBV genotype co-infections. None of the patients received anti-viral therapy or immunosuppressant drugs. A written, informed consent was obtained from each family, and the study protocol was approved by the Southern Medical University Ethics Committee.

HBV DNA Extraction and HBV Genotyping

HBV DNA was extracted from 400 μL of serum by QIAamp UltraSens Virus Kit (Qiagen GmbH, Germany), then re-suspended in 50 μL water and stored at −20°C until analysis. HBV genotypes, including C/D recombinant, were initially assigned using the PCR based restriction fragment length polymorphism (RFLP) methods described previously [9], [8].

Cloning of Mixed Infection Samples

For samples with mixed genotype infections, PCR cover HBV S gene (nt136-1110) was performed using the primers and thermocycling conditions described by Sugauchi et al [10]. For samples needing further recombination analysis, PCR was performed using the primers and thermocycling conditions described by Günther to obtain full-length HBV genome [11]. Alternatively, a nested PCR was used to produce two overlapping fragments in subjects with low HBV DNA levels as described by Sugauchi et al [12]. The spanning of fragment A cover nucleotides 2813 to 1824, and fragment B included nucleotides 1821 to 237. LA-Taq (TAKARA, Japan) and high-fidelity polymerase COD-FX (TOYOBO, Japan) were used to produce amplimers for cloning and direct sequencing respectively. Finally, Fragment C (HBV nt56-nt1824) was obtained from a PCR amplification of Y2 HBV-DNA to which an aliquot of genotype B HBV-DNA had been added. The purpose of this experiment with in-tube control of genotype B was to determine if the recombinant clones were being generated during the PCR amplification. PCR products were gel-purified and cloned into the PMD19-T vector (TAKARA, Japan) according to the manufacturer's instructions, and used to transform JM109 competent cells (TAKARA, Japan). A minimum of 15 clones were sequenced from subjects with a mixed-strain infection and three clones were sequenced from family members with a single-strain infection. All sequencing of clones and PCR products was performed by Invitrogen Ltd. (Shanghai, China).

Phylogenetic and Recombination Analysis

Genotypes of clones were determined by phylogenetic tree analysis and recombination analysis. The sequences were assembled using SeqMan II software (DNAStar Inc.). Sequence alignments were performed using ClustalW and confirmed by visual inspection. Phylogenetic trees were constructed by the neighbour-joining (NJ) method (Saitou & Nei, 1987). To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. A phylogenetic tree analysis of HBV strains isolated from the mixed infection family was compared with reference strains from GenBank. Accession numbers are indicated on the tree. Bootstrap values are shown along each main branch. The lengths of the horizontal bars indicate the number of nucleotide substitutions per site. The regions included in the analysis were the same with fragment A, B and C or a little shorter. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura, Peterson, Stecher, Nei, and Kumar 2011).

Recombination signals were initially detected by RDP3.β.4 software [13,14]. Bootscan, Geneconv and Siscan were used. The highest acceptable P-value was 0.05. Bootscan and Siscan window sizes were 300 bp, step size was 30, replicates for 100 times. A genotype F sequence (GenBank accession numbers is X75658 and X75663) was used as external reference. The precise map of recombination was determined by split phylogenetic tree and alignment. Split phylogenetic trees were constructed by the method same as above. In alignment, each clone was compared to reference C2, D1 and CD1 consensus sequences. We then inspected the alignments to determine the identical crossover sequences around the breakpoint within which the recombination occurred.

Accession Number of the Sequences

GenBank accession number of reference sequences of HBV genotype C2, D1, CD1 and CD2 are indicated in phylogenetic tree. Accession Numbers of Y2 clones are JX036326-JX036359.

Results

Mixed-genotype Infections in HBV Cluster Families

Different HBV genotypes were found in three families among 15 families. The flow of participants in the study and family trees of families with mixed genotypes/subgenotypes of HBV infection are shown (Figure 1).

Family V had infected members across two generations and two genotypes: In 2006, the mother (V1W) and daughter (V2F) were infected with subgenotype D1 while the son (V2M) had a CD1 recombinant. In 2007, the daughter (V2F) had subgenotype D1 while other family members had HBV DNA levels below the detection limit of the nested PCR assay.

Family Q had infected members across three generations and two genotypes/subgenotypes. In 2006, the grandmother (Q1W) and grandson (Q3M) were infected with CD1 recombinant while father (Q2) and granddaughter (Q3F) had mixed infections of genotype C2 and CD1 recombinants. In 2007, the same genotypes were detected in all family members except that the granddaughter (Q3F) had an HBV DNA level below the detection limit of the nested PCR assay.

Family Y had affected members across three generations and three genotypes/subgenotypes. In 2006, the grandfather of family Y (Y1) was infected with genotype C2 while grandmother (Y1W) had mixed infections of CD1 and C2. Mother (Y2W) and granddaughter (Y3F) were infected with the CD1 recombinant. Father (Y2) had triplicate infections of genotype C2, D1 and CD recombinant. Grandson's (Y3M) serum was unavailable. In 2007, the grandfather (Y1) and mother (Y2W) had HBV DNA levels below the detection limit while the grandmother (Y1W) and granddaughter (Y3F) had genotype CD1. Father (Y2) and grandson (Y3M) had genotype D1.

Phylogenetic Analysis of Family Y, Family Q and Family V

A phylogenetic tree constructed from HBV nt 36-1110 from the clones of family Y is given (Figure 2A). The clones (dotted) of family Y exhibits three clusters on genotype C2, D1 and CD1.

The phylogenetic tree construct from HBV nt136-1110 from the clones of families Q and V is given (Figure 2B). The clones of

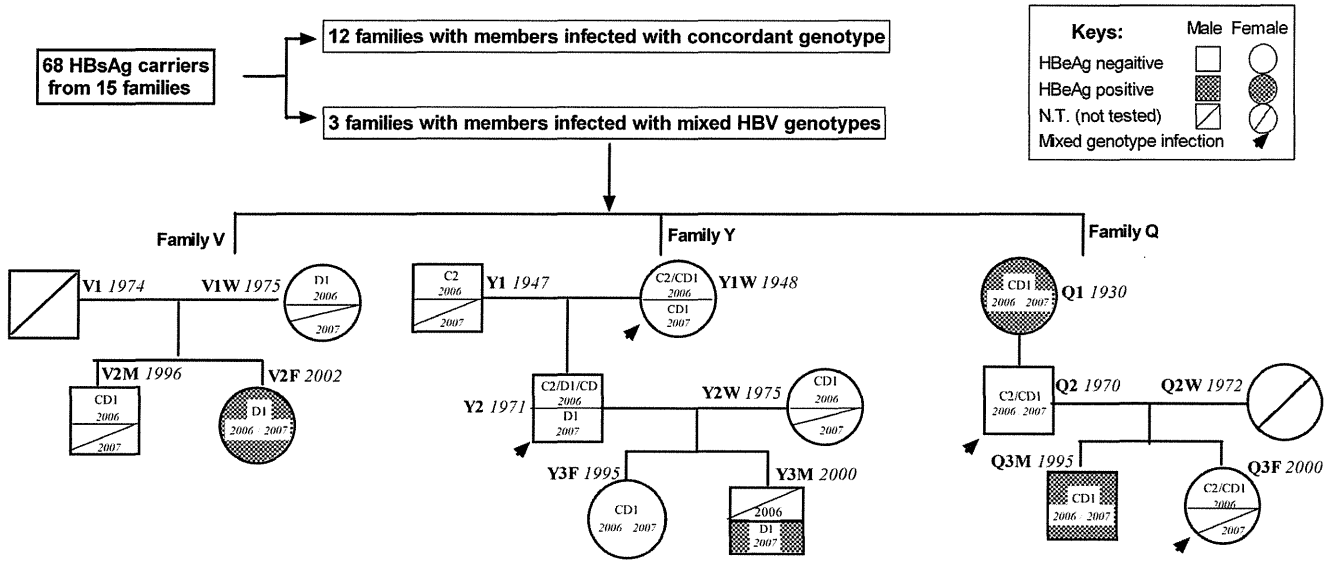

Figure 1. Flow of participants in the study and family trees of family with mixed genotypes/subgenotypes HBV infection. Circles and rectangles correspond to female and male individuals, respectively. Family name and birth date of the patients are indicated beside the circles and rectangles. Subgenotype and the year of blood sampling are indicated inside the circles and rectangles. Family V with affected members across two generations and two genotypes/subgenotypes. Family Q with affected members across three generations and two genotypes/subgenotypes. Family Y with affected members across three generations and three genotypes/subgenotypes. Specially, father (Y2) of family Y with triplicate infection of genotype C, D and CD recombinant in 2006. N.T: Not tested for HBV DNA level below the detection limit of the nested PCR assay or no serum was available.
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family Q (indicated by black dots) exhibit two clusters of subgenotypes C2, and CD1. The clones (indicated by black triangles) from family V exhibit two clusters of subgenotypes D1 and CD1.

A phylogenetic tree constructed from HBV nt 36-1110 of novel recombinants clones of Y2 is given in Figure 2C. The dotted clones are from Y2. The topology of phylogenetic tree with recombinants is totally different from typical trees. Recombinant sequences blurred the typical branch,in other words, blurred the typical genotype.

Recombination and Crossover Analysis of Quasi-species of Y2

Results of recombination analysis of Y2 clones are as bellow: Three kinds of analytical methods certificated the same recombination map. The initial pictures of the three methods were all provided as supplemental figures. Recombination events detected by RDP software are shown in Figure S1, S2, and S3. Split phylogenetic trees constructed by MEGA software are shown in Figure S4, S5, and S6, (clone number and fragment used to construct tree are indicated beside each tree). Sequence alignments are shown in Figure S7, S8, and S9.

The region where recombination breakpoints had the highest probabilities was recognized as crossover region, which is a region that one parental genotype switches to another. Upstream sequence of crossover region will have specific mutation of one genotype but with no specific mutation of another, downstream just opposite. At the same time, these two genotypes should share same sequence at crossover region. We indicated the crossover region in direct alignment by black bars in Figure S3 initially and marked it in recombination map by colorful bars in Figure 3A and black bars in Figure 3B. The clonal sequences of 2006 showed 17 unique crossover regions in fragments A, B and C. We could not identify any common motif within these sequences that might suggest a common mechanism for crossovers in the HBV. The size of switch region share the same sequence are different in different strains, from 6–174 bp (6 bp for Y2M-2 clone in Figure S7 and 174 bp for Y2M-29 clone in Figure S8).

To illustrate the recombination map in a simple way. An abbreviated alignment of fragment A, B and C are shown in Figure 3B. Green and pink bars indicated the genotype C2 and D1 respectively. Black bars showed the crossover region. The aligned sequences provide a snapshot of the recombinant HBV strains. Genotype C2, D1 and CD1 recombinant clones of Y2 were all used as parental sequences to recombine with each other to form new recombinants. A series of novel recombinants were found in three fragments.

In 15 clones of fragment A, there were five genotype C (Y2-6,9,13,14,15,); two genotype D (Y2-11,12); one CD1 (Y2-10) and seven novel different C/D recombination (Y2-1,2,4,7,8,3,5).

In 16 clones of fragment B, there were four genotype C (Y2-23,71,78,75); seven genotype D (Y2-25, 27,79,76,72,22,210); one CD1 (Y2-29) and four novel C/D recombinants (Y2-212,21 73,77).

Of the 56 clones of fragment C(in which genotype B HBVDNA were added as an in-tube control to exclude the recombination by PCR procedure), there were 32 pure genotype B clones; nine genotype C clones(Y2-B10,B5,B8,B9,B13,B16.B17.B18,B24); five genotype D clones(Y2-B22,B3,B4,B21,B23), two CD1 clones (Y2-B1,B11) and eight novel C/D recombinants (Y2-B6,B7,B14,B15,B19,B2,B12,B20). No recombinants of genotype B were found.

Discussion

Recombination is one of the major mechanisms contributing to the evolution of retroviruses [15]. Since the HBV has a reverse transcription step in its life cycle, it is conceivable that recombination also contributes to diversity in HBV genomes. Although just four cases were observed with mixed genotype

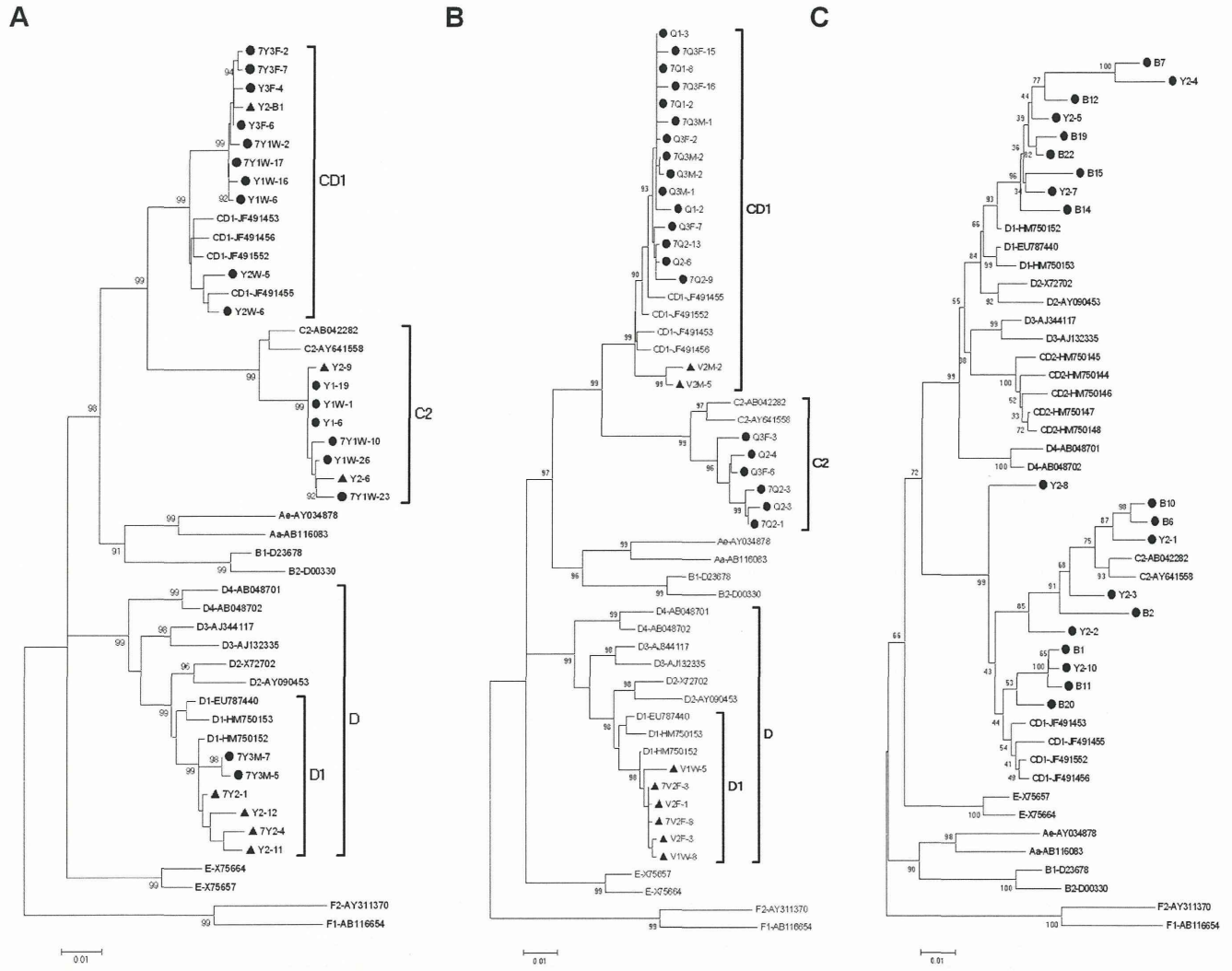

Figure 2. Phylogenetic tree construct by HBV nt 136-1110. (**A**) clones of family Y. Solid dots indicate the clones from Y1,Y1W,Y2W,Y3F and Y3M; Solid triangles indicate the clones from Y2. Family names starting with number 7 means the samples collected in 2007 otherwise in 2006. Novel recombinants of Y2 were excluded from the phylogenetic tree. (**B**) clones of family Q and family V. Solid dots indicate the clones from family Q; Solid triangles indicate the clones from family V. A family name starting with number 7 means the samples collected in 2007, otherwise, in 2006. (**C**) Novel recombinant clones of Y2. Solid dots indicate the clones from Y2.
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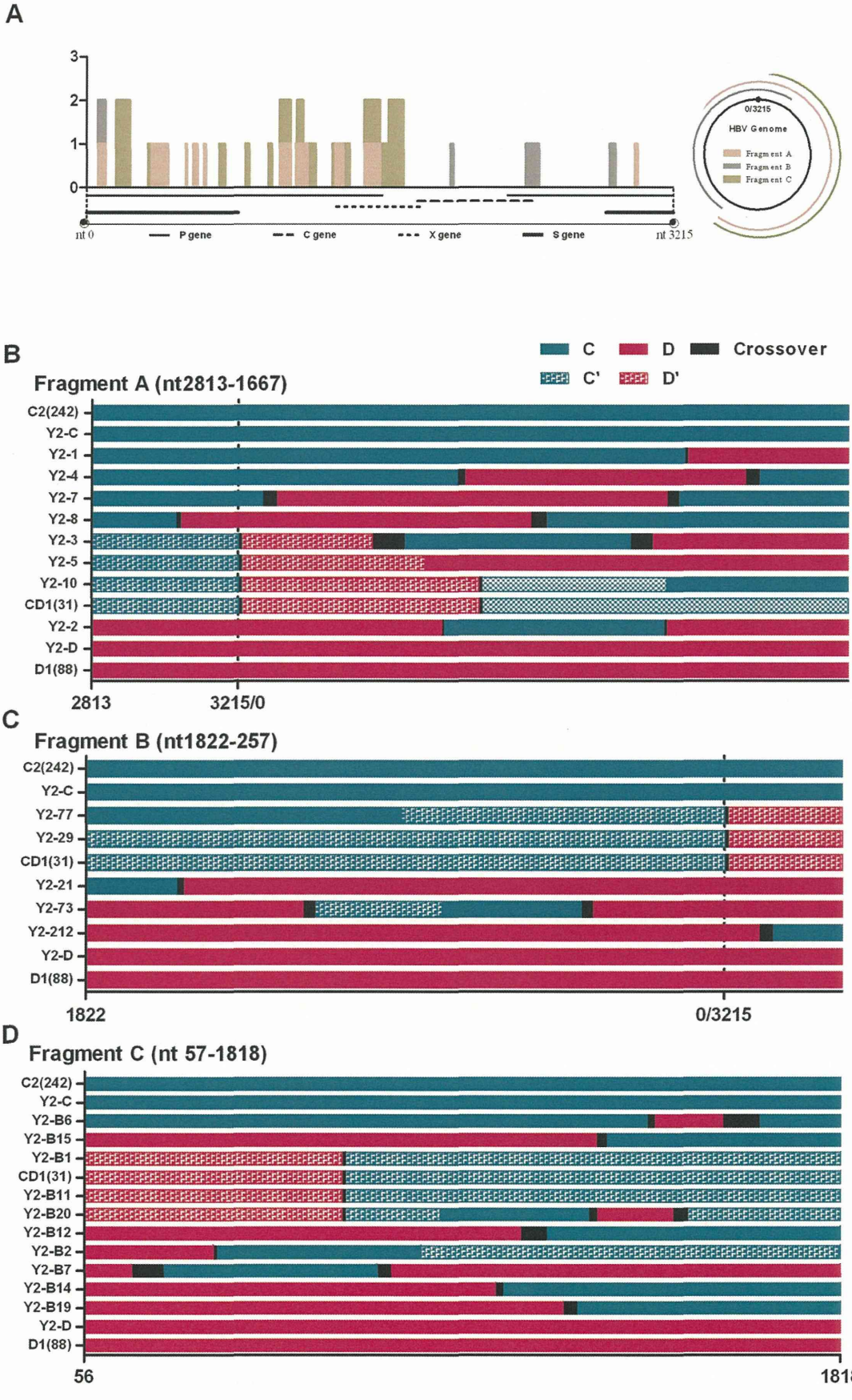
infections, we obtained a snapshot of naturally occurring HBV recombinants generated in the absence of selection and after selection. Our result showed direct evidence of HBV recombination, with new information of recombining crossovers compared with similar studies [16,17,18,19].

The recombination analysis of Y2 quasi-species showed variable types of recombinant between genotype C2, D1 and CD1 in 2006. Some studies show that hotspots of recombination most on the boundary of ORFs [12,20]. Our results showed that two or more strains of HBV can recombine with each other at any region along the genome. Crossover regions can be hundreds or just several base pairs, The length of crossover region is depends on the location of it on HBV genome. If it is located in a conserved HBV region, for another word, where many different genotypes share the same sequence, the length of crossover region may be long. If it is located in a non-conserved region, it may be very short. At the same time, we found that the crossover region distributed totally at random on HBV genome. Consistent with our results, *in vitro* evidence showed the initial recombination events in a laboratory system of MHV were almost entirely randomly distributed along the sequence [21]. It was only after passage through cell culture, with the opportunity for selection to remove less fit variants, that crossover sites became "localized" to just a small area of the region examined. Crucially, they also suggested initial products of recombination may go undetected because of the action of strong purifying selection which will remove new, deleterious combinations of mutations. The conclusion is therefore an interpretation for the genotype change of Y2. The Y2 presented multiple strain infections of C2/D1/CD1 and many new recombinants with no obvious dominant genotype strain in 2006. After 18 months, however, all the type C2 and CD recombinant strains disappeared while the D strain became dominant. A similar case of mixed HBV genotype infection in which one genotype was lost and another prevailed was previously described in patients with HBeAg seroconversion [4,22].

Epidemiologically, HBV genotype CD1 and C2 are the most common strains in ethnic minorities of northwest China with CD2 and D1 as minor strains. Precise mapping of recombination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0
nt 0
P gene
C gene
X gene
S gene
nt 3215
0/3215
HBV Genome
Fragment A
Fragment B
Fragment C
B
C
D
Crossover
C'
D'
Fragment A (nt2813-1667)
C2(242)
Y2-C
Y2-1
Y2-4
Y2-7
Y2-8
Y2-3
Y2-5
Y2-10
CD1(31)
Y2-2
Y2-D
D1(88)
2813
3215/0
C
Fragment B (nt1822-257)
C2(242)
Y2-C
Y2-77
Y2-29
CD1(31)
Y2-21
Y2-73
Y2-212
Y2-D
D1(88)
1822
0/3215
D
Fragment C (nt 57-1818)
C2(242)
Y2-C
Y2-B6
Y2-B15
Y2-B1
CD1(31)
Y2-B11
Y2-B20
Y2-B12
Y2-B2
Y2-B7
Y2-B14
Y2-B19
Y2-D
D1(88)
56
1818

Figure 3. Alignment and recombination crossover regions found in Y2 clones. (**A**) Frequency and distribution of the recombination crossover regions found in Y2 clones along the HBV genome. The bars indicate the number of clones (y axis) showing recombination crossover regions at each site. The 1-3215 of x axis was consistent with the nt1-3215 of HBV genome. Different colors represent the sites find from clones of different PCR region: pink bars for fragment A, grey bars for fragment B and green bars for fragment C. (**B**) Alignment of fragment A (HBV nt 2813-0-1667). Y2-1′12: clones from fragment A of Y2 patients. (**C**) Alignment of fragment B (HBV nt 1822-0-257). Y2-21′212: clones from fragment B of Y2 patients. (**D**) Alignment of fragment C (HBV nt 57-1818) of Y2 clones. Y2-B1′B22: clones from fragment C of Y2 patients. The number on the x axis was consistent with the site of nucleotides of HBV genome. Solid green lines are genotype C2, solid pink lines are genotype D1, speckled green lines are the C2 component of genotype recombinant CD1 and speckled pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242) is the consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88) is the consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33) is the consensus sequence formed by CD1 recombinant sequences from GenBank.
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suggests C2 and D1 are parental sequences of CD1 and CD2 recombinants. Virological differences among HBV genotypes were demonstrated in vitroand in CHiM mice, with genotype C having a higher replication capacity than D [23]. Why does the replication-deficient genotype D virus predominate over replication-competent genotype C? As mixed HBV infections together with recombination are rare, we have little knowledge about i this situation. On the one hand, we know little about host impact on different genotypes and recombinants. On the other hand, we know little about interference and competition in the quasi-species of mixed infection. In vitro results showed the replication capacity of individual clone, exclude the influence of host and other strains of quasi-species. An example from a ChiM mice study showed that monoinfection of HBV/G in ChiM mice display a very slow replication while coinfection with HBV/A remarkably enhanced the replication of HBV/G. The replication of HBV/G is heavily dependent on coinfection with other genotypes. When HBV/G superinfected on other genotypes, a rapidly takes over of HBV/G from original genotype were observed, though they are indispensable [24]. This study confirms that in a mixed infection system of different genotypes, the replication capacity of a genotype may be different from that of monoinfection. At the same time, replication capacity is not the only factor to influence which strain will become dominant. Variable recombinants found in our study may be mechanistically capable of genetic exchange, but strong selection guaranteed the elimination of hybrid genomes. The mechanism of selection in mixed infection also needs more investigation.

We found mixed HBV genotypes infection with many novel recombinants at one point in time, but just one genotype was found 18 months later. This may indicate that the detectable mixed infection and recombination has a limited time window due to the sensitivity of detection or strong selection power of the host. That's why in most studies, we can identify a major genotype in one patient. Even so, evolutionarily visible and invisible recombination of HBV could occur and play an important role by generating genetic variation or reducing mutational load. However, this study had limitation, because recombination signals were detected by RDP3 software and confirmed by split phylogenetic tree and alignment, indicating the recombinant or recombinant-like form should depend on the software. If we use another software, the results might be different.

Studies of HBV in endemic areas throughout the world have resulted in large numbers of full genome sequences available for phylogenetic analysis enabling the identification of novel, mosaic HBV genomes that appear to be the result of recombination between previously known sequences [7,25,26]. One of the most comprehensive analyses of putative HBV inter-genotype recombinants showed the existence of 24 phylogenetically independent HBV genomes involving all known human genotypes [27]. Some of these recombinants are unique to individual subjects, but some undergo expansion in specific populations and become recognized as new genotypes or subgenotypes [12,28,29,30]. Four stages in the process of generating popular HBV recombinant genomes should be recognized. The first stage is the co-circulation of different HBV strains or genotypes in the same geographic area. The second is the existence of individuals who have been infected with more than one strain of HBV. The third is the generation of a novel recombinant strain(s) within an individual. The fourth is the selection of a recombined strain with the ability to replicate and be transmitted. Our data show the natural process of the formation and selection of recombination though the recombinant strains of Y2 that appeared in 2006 that were all removed from samples in 2007.

By using phylogenetic trees and homology calculations, HBV variants infecting humans are currently classified into ten genotypes that differ from each other in nucleotide sequence by 7.5 to 13% [2,3]. There are some characteristic length differences between the genotypes that facilitates their detection and discrimination. However, as shown in Figure 2, existence of a recombinant makes the topology of the phylogenetic tree totally different from one with no recombinant. Recombinant strains obscured the definition of genotypes. Based on the algorithm creating a phylogenetic tree, sequences with high homologues cluster together. With the same logic, recombinants always clustered with the backbone parental sequence, in other words, with which they have high similarity with the larger proportion of the recombination region. Therefore, recombinants always seem to be a subgenotype of their backbone parental sequence. Similar to Y2-8 clone in Figure 2C, for recombinants with similar proportion of both parental genotypes, the sequence shows a divergent trend different from both parental genotypes.

Based on phylogenetic topology changes of different regions of HBV, it was hypothesized that some of the genotypes that are conventionally regarded as "pure," actually were recombinant. Genotype E strains show evidence of recombination with genotype D at 1950–2500. new reported genotype "I" actually belongs to genotype C. Furthermore, Subgenotype Ba possesses the recombination with genotype C at 1740 to 2485 [31,32,33]. Recombinants comprising regions with different histories have important implications for the way we think about HBV evolution. It means that there is no single phylogenetic tree that can describe the evolutionary relationships between genotypes.

In conclusion, mixed HBV genotypes infection with many novel recombinants at one point in time ended up with just one genotype 18 months later in this study. This may indicate that the detectable mixed infection and recombination have a limited time window due to the sensitivity of detection or strong selection power of the host. Also, as the recombinant or recombinant-like nature of HBV precludes the possibility of a "true" phylogenetic taxonomy, a new standard may be required for classifying HBV sequences.

Supporting Information

Figure S1 Recombination map of fragment A created by RDP software.
(TIF)

Figure S2 Recombination map of fragment B created by RDP software.
(TIF)

Figure S3 Recombination map of fragment C created by RDP software.
(TIF)

Figure S4 Split phylogenetic trees constructed by MEGA software. clone number and fragment used to construct trees are indicated beside each tree.
(TIF)

Figure S5 Split phylogenetic trees constructed by MEGA software. clone number and fragment used to construct trees are indicated beside each tree.
(TIF)

Figure S6 Split phylogenetic trees constructed by MEGA software. clone number and fragment used to construct trees are indicated beside each tree.
(TIF)

Figure S7 Alignment of fragment A(HBV nt 2813-0-1667)of Y2 clones. Deep green lines are genotype C2, deep pink lines are genotype D1, light green lines are the C2 component of genotype recombinant CD1 and light pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242): consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88): consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33): consensus sequence formed by CD1 recombinant sequences from GenBank. Y2-1'12: clones from fragment A of Y2 patients.
(DOC)

Figure S8 Alignment of fragment B(HBV nt 1822-0-257) of Y2 clones. Deep green lines are genotype C2, deep pink lines are genotype D1, light green lines are the C2 component of genotype recombinant CD1, light pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242): consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88): consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33): consensus sequence formed by CD1 recombinant sequences from GenBank. Y2-21'212: clones from fragment B of Y2 patients.
(DOC)

Figure S9 Alignment of fragment C(HBV nt 57-1818) of Y2 clones. Deep green lines are genotype C2, deep pink lines are genotype D1, light green lines are the C2 component of genotype recombinant CD1, light pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242): consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88): consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33): consensus sequence formed by CD1 recombinant sequences from GenBank. B1B22: clones from fragment C of Y2 patients.
(DOC)

Author Contributions

Conceived and designed the experiments: ZW MM JH. Performed the experiments: BZ ZW. Analyzed the data: BZ JY JS. Contributed reagents/materials/analysis tools: HL YT. Wrote the paper: BZ YT.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Sequential immunological analysis of HBV/HCV co-infected patients during Peg-IFN/RBV therapy

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Abstract
Background The immunopathogenesis of dual chronic infection with hepatitis B virus and hepatitis C virus (HBV/HCV) remains unclear. The in vivo suppressive effects of each virus on the other have been reported. In this study we aimed to analyze the virological and immunological parameters of HBV/HCV coinfected patients during pegylated interferon/ribavirin (Peg-IFN/RBV) therapy.
Methods One patient with high HBV-DNA and high HCV-RNA titers (HBV-high/HCV-high) and 5 patients with low HBV-DNA and high HCV-RNA titers (HBV-low/HCV-high) were enrolled. Twenty patients monoinfected with HBV and 10 patients monoinfected with HCV were enrolled as control subjects.. In vitro cultures of Huh 7 cells with HBV/HCV dual infection were used to analyze the direct interaction of HBV/HCV.
Results Direct interaction of HBV clones and HCV could not be detected in the Huh-7 cells. In the HBV-high/HCV-high-patient, the HCV-RNA level gradually declined and HBV-DNA gradually increased during Peg-IFN/RBV therapy. Activated CD4- and CD8-positive T cells were increased at 1 month of Peg-IFN/RBV-therapy, but HBV-specific IFN-γ-secreting cells were not increased and HBV-specific interleukin (IL)-10 secreting cells were increased. The level of HBV- and HCV-specific IFN-γ-secreting cells in the HBV-high/HCV-high-patient was low in comparison to that in the HBV- or HCV-monoinfected patients. In the HBV-low/HCV-high-patient, HCV-RNA and HBV-DNA rapidly declined during Peg-IFN/RBV therapy. Activated CD4- and CD8-positive T cells were increased, and HBV- and HCV-specific IFN-γ-secreting cells were also increased during Peg-IFN/RBV-therapy.
Conclusion The immunological responses of the HBV-high/HCV-high patient were low in comparison to the responses in HBV and HCV monoinfected patients. Moreover, the response of immune cells in the HBV-high/HCV-high patient during Peg-IFN/RBV therapy was insufficient to suppress HBV and HCV.

Keywords Dual infection · HBV · HCV · Immunopathogenesis

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Introduction

Hepatitis B virus (HBV) and Hepatitis C virus (HCV) are noncytopathic viruses that cause chronic hepatitis and hepatocellular carcinoma (HCC) [1, 2]. HBV now affects more than 400 million people worldwide, and persistent infection develops in ~5 % of adults and 95 % of neonates who become infected with HBV [3]. HCV infects about 170 million people worldwide and is a major cause of chronic hepatitis, cirrhosis, and HCC [4]. Some groups have mentioned that dual infection with HBV/HCV is not uncommon in Asian patients [5, 6]. The prevalence of patients with dual HBV/HCV infection is approximately 10–15 %, although it likely differs among countries [7–9]. Co-infection with HBV/HCV has been associated with severe liver disease and frequent progression to cirrhosis [10]. Moreover, a significantly higher incidence of HCC and liver-related mortality was noted in patients with HBV/HCV co-infection [11, 12]. However, some groups reported, based on a meta-analysis, that dual infection with HBV/HCV did not increase the risk of HCC [13, 14]. These contradictory reports could be explained by the rarity of dual infection with HBV/HCV in patients without clinically evident liver disease. It might be difficult to estimate the hepatocarcinogenic risk of dual infection compared with that of either infection alone in such clinical settings [15].

An inverse relationship in the replicative levels of the two viruses has been noted, suggesting direct or indirect effects in vivo [16]. More recently, some groups have reported, using an in vitro infection system, that there is little direct interaction of HBV/HCV in coinfected hepatocytes [17, 18]. Therefore, the viral interference observed in coinfected patients is probably due to indirect mechanisms mediated by the innate and/or adaptive host immune responses.

The cellular immune response to HBV and HCV plays an important role in the pathogenesis of chronic hepatitis, cirrhosis, and HCC [19–21]. Hyporesponsiveness of HBV- or HCV-specific T-helper 1 cells and excessive regulatory function of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) in peripheral blood have been shown in patients with chronic hepatitis B and C [22–34]. Recently, we reported that HBV replication stress could enhance the suppressive activity of Tregs via TLR2 [35]. However, little is known about the immunopathogenesis of HBV/HCV dual infection.

Dual infection can be classified into several groups (e.g., group A: HBV active and HCV active; group B: HBV inactive and HCV active; and group C: HBV active and HCV inactive) [36]. HCV is reported to be the dominant virus in HBV/HCV dual infection, but the dominance of either virus might be due to the genotypes of each virus and/or ethnic differences that could affect the proliferative activity of the viruses [36]. In this study, we investigated immunopathogenesis in a group A patient and in group B patients during therapy with pegylated interferon-α2b (Peg-IFN-α2b) plus ribavirin.

Patients, materials, and methods

Patients

One patient with high HBV-DNA and high HCV-RNA titers (HBV-high/HCV-high; patient A) and 5 patients with low HBV-DNA and high HCV-RNA titers (HBV-low/HCV-high) were enrolled (one of these patients, whose results were analyzed in detail, was termed patient B; see findings below in the "Results"). Twenty patients monoinfected with HBV and 10 patients monoinfected with HCV were enrolled as control subjects. None of the patients had liver disease due to other causes, such as alcohol, drugs, congestive heart failure, or autoimmune diseases. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2006-194). Written informed consent was obtained from all the participants enrolled in this study. Participants were monitored for two years. At each assessment, patients were evaluated by biochemical laboratory tests, immunological analysis, and virological tests. Liver histology was analyzed at the start of Peg-IFN/RBV therapy by using laparoscopic liver biopsy samples and by employment of the METAVIR score.

Detection of interleukin (IL)-28B polymorphism

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using an automated DNA isolation kit. Then polymorphism of IL-28B (rs8099917) was analyzed using real-time polymerase chain reaction (PCR) (TaqMan SNP Genotyping Assay, Applied Biosystems, CA, USA). Detection of the IL-28B polymorphism was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2010-323).

Isolation of peripheral blood mononuclear cells (PBMCs) and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by means of Ficoll-Hypaque density gradient centrifugation (Amersham Bioscience, Uppsala, Sweden). PBMCs were stained with CD3, CD4, CD8, CD19, CD25, CD40, CD56, CD86, HLA-DR, NKG2D, and isotype control antibodies (Becton Dickinson, NJ, USA) for 15 min on ice to analyze the frequency

of $CD3^+CD4^+HLA\text{-}DR^+$ cells, $CD3^+CD8^+HLA\text{-}DR^+$cells, $CD4^+CD25^+$ Tregs, $CD3^-CD16^-CD56^{high}$natural killer (NK) cells, and $CD3^-CD16^+CD56^{dim}$ NK cells. The frequencies of the immune subsets were analyzed by flow cytometry using FACS Canto-II (Becton Dickinson, NJ, USA).

ELISPOT assay

The detection of IFN-γ and IL-10 was performed using an ELISPOT Set (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Cultures of PBMCs were established in triplicate on round-bottomed 96-well plates for all time points investigated, at a concentration of 3×10^5 cells per well in 100 µl RPMI 1640 containing 10 % fetal bovine serum (FBS). Positive spots were detected using an automated counting machine.

Detection of HBV-DNA and determination of HBV genotype

DNA was extracted from 100 µl of serum using SMITEST EX-R&D (Medical & Biological Laboratories, Nagoya, Japan) and dissolved in 20 µl of nuclease-free distilled water. The DNA preparation thus obtained (10 µl) was subjected to nested PCR with primers targeting the S gene of the HBV-DNA, as described previously [37]. Briefly, first-round PCR was carried out for 35 cycles (98 °C for 10 s, 55 °C for 15 s, and 72 °C for 1 min, with an additional 7 min in the last cycle) in the presence of PrimeSTAR HS DNA Polymerase (TaKaRa Bio, Shiga, Japan) and primers HB095 (sense, 5′-GAG TCT AGA CTC GTG GTG GAC-3′) and HB184 (antisense, 5′-CGA ACC ACT GAA CAA ATG GCA CCG-3′), for 25 cycles. This was followed by a second-round PCR consisting of 25 cycles using the same conditions as in the first round, with primers HB097 (sense, 5′-GAC TCG TGG TGG ACT TCT CTC-3′) and S2-2 (antisense, 5′-GGC ACT AGT AAA CTG AGC CA-3′). The HBV genotype was determined by phylogenetic analysis of the S gene sequence (437 nt) of the HBV isolates.

Detection of HCV RNA

RNAs were extracted from 250 µl of serum using TRIzol LS (Invitrogen, Tokyo, Japan). They were divided into two aliquots and each was assayed by reverse transcription (RT)-PCR with nested primers derived from the core region and NS5A interferon sensitivity determining region (ISDR) of the HCV genome. Nested PCR of the core region of the HCV genome was carried out with primers C008 (sense, 5′-AAC CTC AAA GAA AAA CCA AAC G-3′) and C011 (antisense, 5′-CAT GGG GTA CAT YCC GCT YG-3′) in the first round and C009 (sense, 5′-CCA CAG GAC GTY AAG TTC CC-3′) and C010 (antisense, 5′-AGG GTA TCG ATG ACC TTA CC-3′) in the second round. Nested primers that were derived from NS5A-ISDR of the HCV genomes were designed to amplify a 188-bp product with C004 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and C005 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) in the first round, and C006 (sense, 5′-ACC GG A TGT GGC AGT GCT CA-3′) and C007 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) in the second round.

Analysis of nucleotide and amino acid sequences

The PCR products were sequenced directly on both strands using a BigDye Terminator version 3.1 Cycle Sequencing Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Genetyx-Mac ver. 12.2.6 (Genetyx, Tokyo, Japan) and ODEN (version 1.1.1) from the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan) [38]. Sequence alignments were generated using CLUSTAL W (Version 1.8) [39]. The phylogenetic tree was constructed by the neighbor-joining method [40]. The reliability of the phylogenetic results was assessed using 1000 bootstrap replicants [41]. The final tree was obtained with the Njplot program (version 2.2) [42].

Plasmid construction

HBV expression plasmids were constructed by previously published methods. Serum samples were obtained from two patients infected with HBV genotype Bj and two patients infected with HBV genotype C. HBV-DNA was extracted from 100 µl serum using a QIAamp DNA blood kit (QIAGEN, Hilden, Germany). Four primer sets were designed to amplify two fragments covering the entire HBV genome. Amplified fragments were inserted into a pGEM-T Easy Vector (Promega, Madison, WI, USA) and cloned in DH5a competent cells (TOYOBO, Osaka, Japan). Briefly, at least 5 clones of each fragment were sequenced and the consensus sequence was identified and used as a template for 1.24-fold the HBV genome of different genotypes (B1 indicates the genotype Bj35 clone; B2 indicates the genotype Bj56 clone; C1 indicates the genotype C-AT clone; and C2 indicates the genotype C-22 clone). The HCV-JFH-1 strain was provided by Dr. T. Wakita (National Institute of Infectious Diseases, Japan).

HCV and HBV expression in Huh 7 cells

Cell-culture-derived infectious HCV was generated as described previously [43]. The HCV was quantified as

follows: RNA was extracted from the Huh-7 culture supernatant using a QIAamp Viral RNA Kit (Qiagen, Valencia, CA, USA). The HCV RNA was quantified by real-time RT-PCR, using TaqMan EZ RT-PCR Core Reagents (Applied Biosystems) according to the manufacturer's protocol, using the published primers and probe [44]. The filtered (0.45 μm) culture supernatant of HCV-infected Huh-7 cells containing 2×10^8 HCV RNA copies/ml [equivalent to 9.7×10^4 focus-forming units (ffu)/ml] was used for the experiments. To analyze HCV-RNA in the supernatant, Huh-7 cells (2×10^5 cells in a 6-well plate) were infected with JFH-1 (multiplicity of infection [MOI] = 0.01) and after 4 h the cells were washed twice with phosphate-buffered saline (PBS). The supernatants were then collected and the cells were reseeded at 2×10^5 cells per 6-well plate. Then the HBV expression and mock plasmid were transfected by FuGENE6 (Roche Applied Science, IN, USA). The supernatant of the culture medium was collected 72 h after transfection. Quantification of HBV-DNA and HCV-RNA was carried out using real-time PCR.

IFN-α was added 24 h after the transfection of the HBV plasmids, and the supernatant of the culture medium was then collected 48 h after the addition of the IFN-α.

Results

Clinical characteristics of patients A and B

Patient A (high HBV-DNA titer and high HCV-RNA titer)

Patient A was a 44 year-old man with a high aspartate aminotransferase/alanine aminotransferase (AST/ALT) level. The prothrombin time-international normalized ratio (PT-INR) was in the normal range. Patient A had high HBV-DNA titers and high HCV-RNA titers (Table 1). His liver histology was classified as A2/F3 (Fig. 1). The laparoscopic analysis indicated moderate inflammation and intermediate fibrosis. The liver surfaces of the right lobe and left lobe were almost the same phenotype. Polymorphism of IL-28B (rs8099917) was T/G (hetero allele).

Patient B (low HBV-DNA titer and high HCV-RNA titer)

Patient B was a 63 year-old man with a low AST/ALT level. PT-INR was in the normal range. Patient B had low HBV-DNA titers and high HCV-RNA titers. The liver histology was classified as A2/F1 (Fig. 1). The liver surface showed moderate inflammation and was smooth. The polymorphism of IL-28B (rs8099917) was T/T (major homo allele).

Biopsy samples from patients with dual HBV and HCV infection were collected at the main liver centers in Miyagi prefecture. Fifteen HBV/HCV dual-infected patients were found in this study (Supplementary Table 1). Many of these patients had HCV-dominant infection and undetectable levels of HBV replication (10/15 patients). Most of the patients were HB envelope antigen (eAg)-negative and HBe antibody (Ab)-positive (14/14 patients). All HBV/HCV dual-infected patients who had received Peg-IFN-based

Table 1 Background of HBV/HCV dual-infected patients

	Patient A HCV high titer/ HBV high titer	Patient B HCV high titer/ HBV low titer	Normal range
Gender	Male	Male	
Age (years)	44	63	
HCV-RNA	6.5	5.5	log copies/ml
HCV genotype	1b	1b	
HBV-DNA	5.5	3.5	log copies/ml
HBV genotype	C	Bj	
HBe-Ag	129.5	0.5	0–0.9 index
HBe-Ab	0.1	99.3	0–49 %
Total bilirubin	0.7	1.2	0.2–1.2 mg/dl
Direct bilirubin	0.1	0.1	0–0.3 mg/dl
γ-GTP	208	31	8–57 IU/l
AST	138	33	12–30 IU/l
ALT	256	38	8–35 IU/l
Hb-A1c	5.3	5.4	4.3–5.8 %
Glu	103	83	68–106 mg/dl
BMI	25.34	18.75	
T-cho	160	195	128–220 mg/dl
LDL-cho	69	93	70–139 mg/dl
HDL-cho	37	67	36–89 md/dl
WBC	7800	5100	3200–9600/μl
RBC	491	446	428–566 × 10^4/μl
Hb	17.1	14.1	13.6–17.4 g/dl
PLT	169000	176000	155000–347000/μl
PT-INR	0.87	0.96	0–1.15 INR
Liver histology	A2/F3	A2/F1	METAVIR score
IL-28B SNP (rs8099917)	T/G	T/T	

HCV hepatitis C virus, *HBV* hepatitis B virus, e-*Ag* envelope antigen, *e-Ab* envelope antibody, *γ-GTP* γ-guanosine triphosphate, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *Hb* hemoglobin, *Glu* glucose, *BMI* body mass index, *T-cho* total cholesterol, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *PLT* platelets, *PT-INR* prothromin time-international normalized ratio, *IL* interleukin, *SNP* single-nucleotide polymorphism

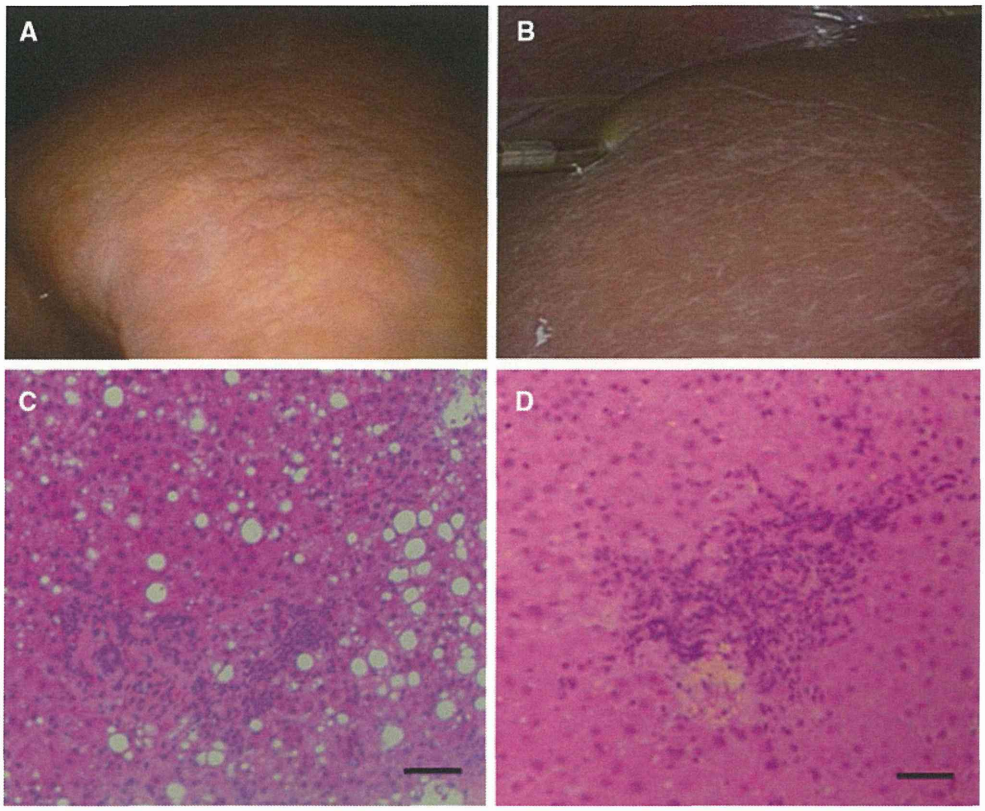


Fig. 1 Laparoscopic liver biopsy. The laparoscopic images of the liver surfaces of patient A (**a**) and patient B (**b**) are shown. Histopathology of patient A (**c**) and patient B (**d**) is also shown. *Bars* = 50 μm

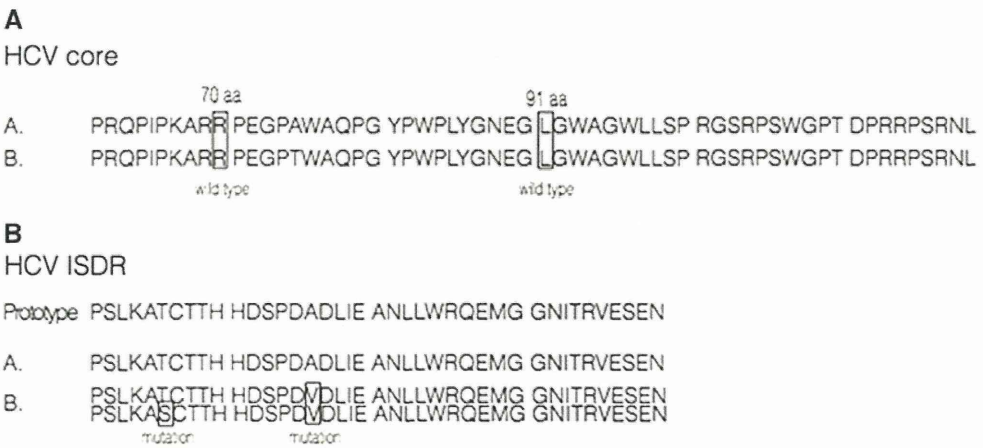


Fig. 2 Virological analysis of hepatitis B virus (HBV) and hepatitis C virus (HCV) in HBV/HCV dual infection. The amino acid sequences of the HCV-core region including core-70 and core-91, which were previously reported as determinants of the sensitivity to pegylated interferon/ribavirin (Peg-IFN/RBV) therapy, in patient A and patient B are shown (**a**). The amino acid sequences of the interferon sensitivity determining region (*ISDR*), which were previously reported as determinants of the sensitivity to IFN, in patients A and B are shown (**b**)

treatment achieved a sustained viral response (SVR) (5/5 patients). These data indicated that HCV-dominant dual-infected patients had good responses to treatment for HCV infection.

Virological analysis of HBV/HCV in patients A and B

The HCV genotype in both patient A and patient B was 1b. The sequences of amino acids in the ISDR region and HCV

core-70 and core-91 amino acids were analyzed by direct sequencing. Both patients had wild-type core-70 and core-91 amino acids (Fig. 2a). None of the mutations of the ISDR region was detected in patient A, but two of the mutations of the ISDR region were detected in patient B (Fig. 2b). The genotypes of HBV in patients A and B were analyzed by direct sequencing and phylogenetic tree analysis. The genotype of HBV in patient A was genotype C, which has been reported as difficult-to-treat HBV. The genotype of HBV in patient B was genotype Bj, which has been reported as easy-to-treat HBV in comparison to genotype C [45–47].

Sequential analysis of biochemical and virological data during Peg-IFN/RBV therapy

Patient A

In patient A, HCV-RNA gradually declined during Peg-IFN/RBV therapy. On the other hand, the HBV-DNA gradually increased during Peg-IFN/RBV therapy (Fig. 3a). The amount of HBeAg started to increase 9 months after the start of Peg-IFN/RBV therapy. HCV-RNA started to increase 12 months after the start of Peg-IFN/RBV therapy, although Peg-IFN/RBV was still being administered up to 18 months after the start of Peg-IFN/RBV therapy (Fig. 3a).

Patient B

In patient B, HCV-RNA and HBV-DNA rapidly declined after the start of Peg-IFN/RBV therapy (Fig. 3b). HCV-RNA could not be detected in peripheral blood 2 months after the start of Peg-IFN/RBV therapy. Peg-IFN/RBV was administered up to 12 months after the start of the Peg-IFN/RBV therapy. The amounts of HBeAb and HBeAg did not change during the Peg-IFN/RBV therapy (Fig. 3b).

Sequential immunological analysis during Peg-IFN/RBV therapy

We analyzed various subsets of immune cells that could affect the immunopathogenesis of HBV/HCV dual infection. NK cells ($CD3^-CD16^-CD56^{high}$ and $CD3^-CD16^+CD56^{dim}$) and NK-T cells ($CD3^+CD56^+CD16^+$, $CD3^+CD56^+CD16^-$ and $CD3^+CD56^-CD16^+$) were analyzed (Supplementary Fig. 1A). The $CD3^-$ gated lymphocytes were separated into 4 groups (a, b, c, and d). For these subsets, (a) indicated the presence of $CD3^-CD16^-CD56^{high}$ NK cells that could produce various cytokines vigorously and had low cytotoxic activity. Subset (b) showed $CD3^-CD16^+CD56^{dim}$ NK cells that had weak cytokine production ability and high cytotoxic activity.

The $CD3^+$ gated lymphocytes were separated into 3 groups (a, b, and c). The activated $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T cells were analyzed (Supplementary Fig. 1B). HLA-DR^+ activated $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T cells could be clearly distinguished by FACS analysis. Additionally, representative dot plots of Tregs and B cells were created (shown in Supplementary Fig. 1C). The frequencies of $CD3^-CD16^+CD56^{dim}$ NK cells, $CD3^+CD16^-CD56^+$ NK-T cells, activated $CD3^+CD4^+$ T cells, and activated $CD3^+CD8^+$ T cells fluctuated similarly during Peg-IFN/RBV therapy in patient A (Supplementary Fig. 1D). Activated T cells were increased at one month of Peg-IFN/RBV therapy, and the above subsets of lymphocytes gradually decreased up to 3 months of Peg-IFN/RBV therapy. After that, these cells gradually increased again up to 9 months of Peg-IFN/RBV therapy. In patient A, after 9 months of Peg-IFN/RBV therapy, these cells had decreased again (Supplementary Fig. 1D). The frequency of Tregs and activated B cells (data not shown) did not change during Peg-IFN/RBV therapy in patient A (Supplementary Fig. 1D). On the other hand, in patient B, the frequencies of $CD3^-CD16^+CD56^{dim}$ NK cells, $CD3^+CD16^-CD56^+$ NK-T cells, activated $CD3^+CD4^+$ T cells, and activated $CD3^+CD8^+$ T cells were increased and sustained during Peg-IFN/RBV therapy (Supplementary Fig. 1E). Five HCV monoinfected patients were analyzed by the same protocol (Supplementary Fig 1F). The mean frequency of various kinds of immune subsets was analyzed (Supplementary Fig 1F). The tendency of immunological reactions during Peg-IFN/RBV therapy in these five patients was similar to that in patient B.

Analysis of HBV- and HCV-specific immune responses

The analysis of HBV- and HCV-specific-immune responses was carried out by ELISPOT assay. Representative spots of IFN-γ are shown in Fig. 4a. In patient A, HCV- and HBV-specific IFN-γ secretion activities were remarkably low in comparison to the IL-10 secretion activity. Moreover, in patient A, the induction of IFN-γ-secreting cells could not be detected after Peg-IFN/RBV therapy, especially in regard to HBV-core specific IFN-γ secretion in PBMCs (Fig. 4b). On the other hand, in patient B, the HBV-core specific IFN-γ-secreting cells were high in comparison to those in patient A (Fig. 4c). Moreover, the induction of IFN-γ-secreting cells could be detected during Peg-IFN/RBV therapy in patient B (Fig. 4c). The mean numbers of IFN-γ- and IL-10-secreting spots in HBV-dominant dual-infected patients, patients with monoinfection with HBV genotype Bj ($HBeAb^+$), Bj ($HBeAg^+$), C ($HBeAb^+$), C ($HBeAg^+$), or HCV genotype 1b are shown in Fig. 4d. In patient A, HB core antigen (HBcAg)-specific IFN-γ secretion was weaker than that in

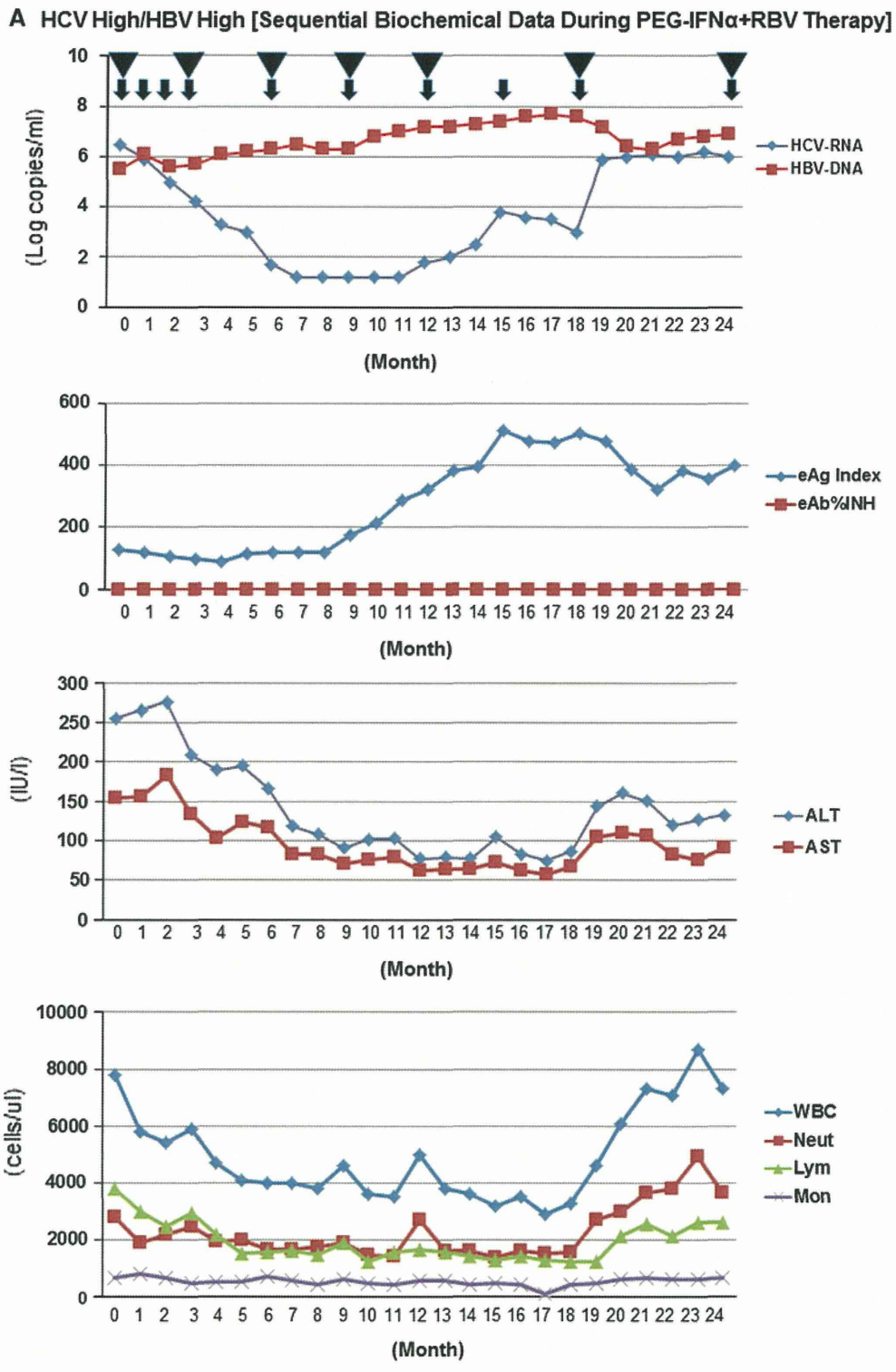
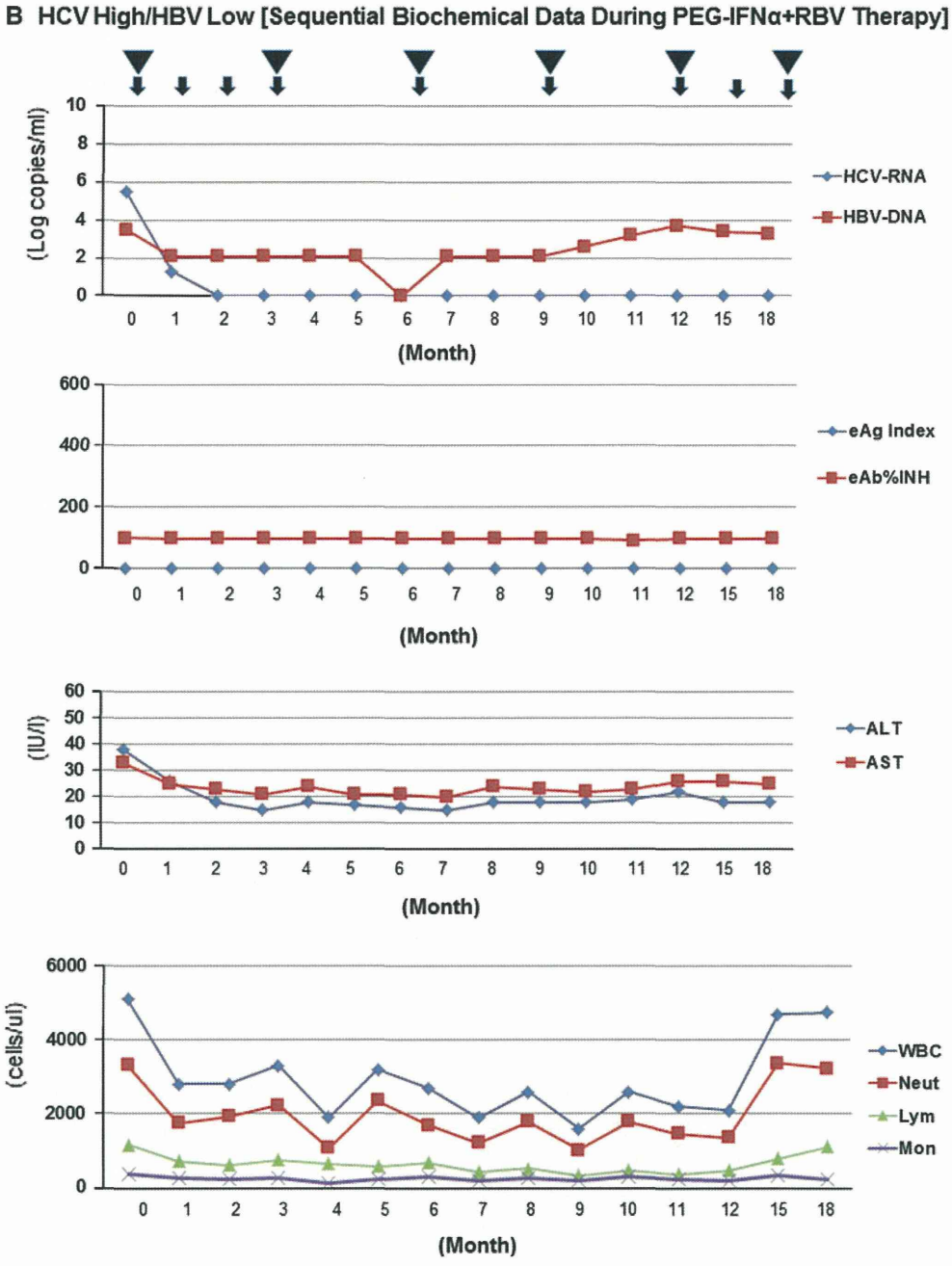


Fig. 3 Sequential biochemical data analysis during Peg-IFN/RBV therapy. The titers of HBV-DNA and HCV-RNA; the amounts of envelope antigen (*eAg*) and envelope antibody (*eAb*), and alanine aminotransferase (*ALT*) and aspartate aminotransferase (*AST*); and the numbers of WBCs, neutrophils (*Neut*), lymphocytes (*Lym*), and monocytes (*Mon*) in patients A (**a**) and B (**b**) are shown in these graphs. *Arrows* indicate the sampling points of FACS analysis. *Triangles* indicate the sampling points of the ELISPOT assay. *INH* inhibition

HBV-genotype C-monoinfected patients who were HBeAg-positive. However, HBcAg-specific IL-10 secretion in patient A was stronger than that in HBV-genotype C monoinfected patients who were HBeAg-positive. These data indicated that the presence of HCV might also suppress the HBV-specific immune response in regard to certain host factors (e.g., in the presence of IL-28B polymorphism, and depending on the body mass index [BMI] and γ-guanosine triphosphate [γ-GTP] level), because the presence of HCV did not suppress the HBV-specific immune response either in patient B or in the patients with dual HCV-dominant infection. Otherwise, we could deny the possibility indicating that

Fig. 3 continued



the certain background of host factors could allow the existence of dual virus actively. These data indicated that HBV-specific IL-10-secreting cells and/or certain kinds of host factors had an important role in HBV- and HCV-specific immune suppression in patient A, but not in patient B.

In vitro analysis of HBV/HCV dual infection

We carried out in vitro analysis of HBV/HCV infection using Huh-7 cells that were susceptible to the HCV-JFH-1 strain and HBV expression plasmids. The amount of the JFH-1 strain did not change with the various kinds of HBV expression plasmids (Fig. 5a). Moreover, the amounts of the various HBV strains did not change in the presence of JFH-1 infection. These data indicated that no direct effect of HBV and HCV could be detected in Huh 7 cells. We carried out experiments to analyze the effect of IFN-α treatment on HCV Huh-7 cells with various kinds of HBV expression (Fig. 5b). In our systems, it appeared that HBV expression could not significantly affect the suppressive effect of IFN-α.

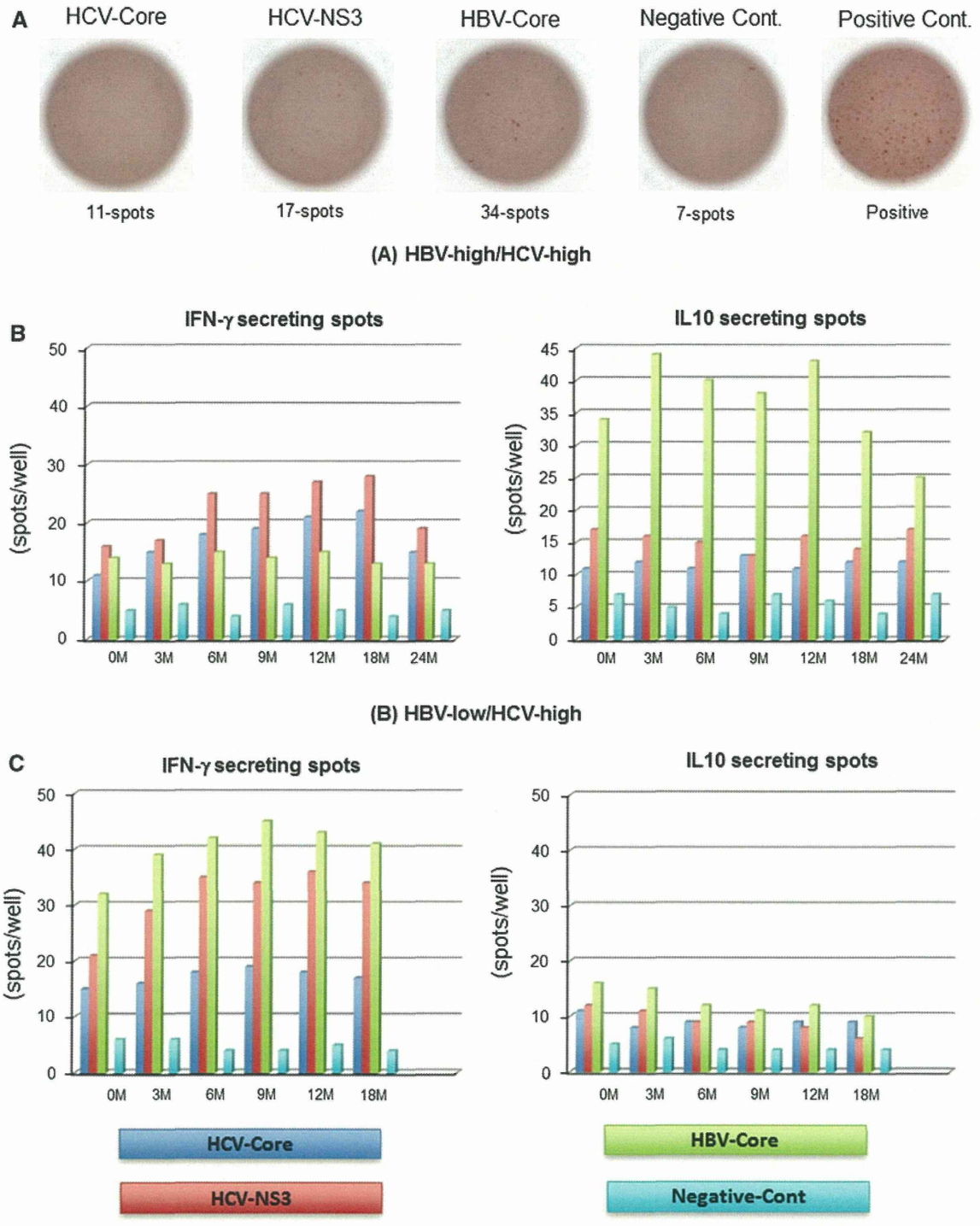


Fig. 4 The sequential analysis of HBV/HCV-specific immune reactions during Peg-IFN/RBV therapy. Representative spots of the ELISPOT assay are shown (**a**). The sequential data of IFN-γ- and interleukin-10 (*IL-10*)- secreting spots in patient A are shown (**b**). The sequential data of IFN-γ- and IL-10-secreting spots in patient B are shown (**c**). Comparison of IFN-γ- and IL-10- secreting spots in patient A before starting therapy, patient B before starting therapy, dual HCV-dominant patients, HCV-monoinfected patients, HBV-Bj ($HBeAb^+$) monoinfected patients, HBV-Bj ($HBeAg^+$) monoinfected patients, HBV-C ($HBeAb^+$) monoinfected patients, and HBV-C ($HBeAg^+$) monoinfected patients (**d**). In these *bar graphs*, the *blue bars* indicate HCV-core specific reaction. The *red bars* indicate HCV-NS3 specific reaction. The *green bars* indicate HBV-core specific reaction. The *aqua blue bars* indicate the negative control (*Cont.*). *Error bars* indicate standard deviations (color figure online)