GGA ACA TGG GGC ATG GAC ATT GAC CCT T-3'), including the T7 promoter sequence, spacer, and the Kozak sequence (underlined) including the initiation codon of the core protein, followed by the partial core sequence and a reverse primer CoreR (5'-CTA TCT AGA CTA ACA TTG GGA TTC CCG A-3') including the termination codon of the core protein. To make templates with G1896A, G1899A, and 1901insA, forward primers CoreK-5A (the underlined sequence of CoreKW was changed to TAGGGCATGG), CoreK-2A (the underlined sequence was changed to TGGGACATGG), and CoreK-1A-2C-6G (the underlined sequence was changed to GGGGCAATGG) were used, respectively. The expressed protein was analyzed with Western blotting using a rabbit polyclonal anti–hepatitis B core antigen (HBcAg) antibody (Dako) as the primary antibody.

Statistical Analysis

Statistical analyses were performed using Mann–Whitney *U* test for comparison of continuous variables between 2 groups. Differences were considered to be statistically significant when $P < .05$.

RESULTS

Characteristics of the Fulminant Hepatitis Patients

The clinical characteristics of the 16 FH patients are shown in Table 1. The mean age was 53.0 years (range, 29–71), and 13 (81%) were male. The mean peak total bilirubin was 14.7, the mean peak alanine aminotransferase was 4932, and the mean lowest prothrombin time was 18.6%. Nine (56%) patients died of fulminant hepatitis. Lamivudine was administered to 4 patients (numbers 12–15), and entecavir was administered to 1 patient (number 16). After 2003, living related liver transplantation has been performed for 4 FH patients, and all of the patients were rescued. Two of them (numbers 12 and 13) showed rapid progression and were considered so-called hyperacute cases, but were rescued with liver transplantation without complications [22]. The HBV isolates from these patients were named BFJT followed by the onset year, excluding 5 cases referred to as FH-1 to FH-5 in a previous report by us [23].

Determination of HBV Genotype

Based on the partial sequences in the S region of HBV isolates from FH patients, a phylogenetic tree was constructed (Figure 1). Of the 16 HBV isolates, 5 (31%) belonged to subgenotype B1/Bj, 6 (38%) belonged to subgenotype B2/Ba, and 5 (31%) were subgenotype C2/Ce. The 5 isolates of subgenotype B2/Ba were grouped into a cluster: these patients were considered to have the same source of infection [23]. In this study, 69% of the FH patients were infected with genotype B HBV, which was much higher than previously reported in Japan (22%–33%) [10, 11]. It was also higher than the reported percentage (21%) of genotype B in acute hepatitis B patients in northeast Japan [24].

Mutation, Insertion, and Deletion in the Core Promoter and Precore Region

The analysis of the partial sequences in the core promoter/precore region showed that there were several mutations in the HBV isolates. The mutations that were reported previously to have an association with FH are shown in Table 2. Because the 5 isolates of subgenotype B2/Ba were almost identical, they were counted as a single strain. The mutations at nucleotides 1753,

Table 1. Characteristics of the Fulminant Hepatitis B Patients

Patient no.	Age/sex	Date of onset	Peak T. Bil (mg/dL)	Peak ALT (IU/L)	Lowest PT (%)	Possible infection source	Liver transplantation	Outcome	HBV isolate name
1	65/M	December 1996	19.2	1764	18.0	Unknown	No	Died	BFJT1996-1
2	29/M	October 1997	8.8	6900	5.0	Unknown	No	Died	BFJT1997-1
3	65/M	February 1999	16.9	2162	22.4	Unknown	No	Died	BFJT1999-1
4	28/F	August 1999	8.9	7120	17.1	Sexual contact	No	Rescued	BFJT1999-2
5	61/M	May 2000	32.8	3750	26.0	Unknown	No	Died	BFJT2000-1
6	69/F	May 2000	10.6	4190	6.0	Iatrogenic	No	Died	FH-1
7	71/M	July 2000	29.0	3530	38.0	Iatrogenic	No	Died	FH-2
8	66/M	October 2000	13.9	6950	10.1	Iatrogenic	No	Died	FH-3
9	50/M	December 2000	13.3	13 420	10.0	Blood transfusion	No	Died	BFJT2000-2
10	71/F	December 2000	17.0	3380	27.0	Iatrogenic	No	Rescued	FH-4
11	60/M	February 2001	6.4	10 290	12.7	Iatrogenic	No	Died	FH-5
12	45/M	July 2003	11.8	6450	9.3	Sexual contact	Yes	Rescued	BFJT2003-1
13	34/M	August 2003	9.5	7150	9.0	Sexual contact	Yes	Rescued	BFJT2003-2
14	32/M	July 2005	10.8	278	22.1	Sexual contact	Yes	Rescued	BFJT2005-1
15	38/M	August 2006	5.1	728	39.8	Unknown	No	Rescued	BFJT2006-1
16	64/M	April 2009	20.5	857	24.7	Carrier	Yes	Rescued	BFJT2009-1

NOTE. ALT, alanime aminotransferase; HBV, hepatitis B virus; PT, prothrombin time; T. Bil, tital bilirubin.

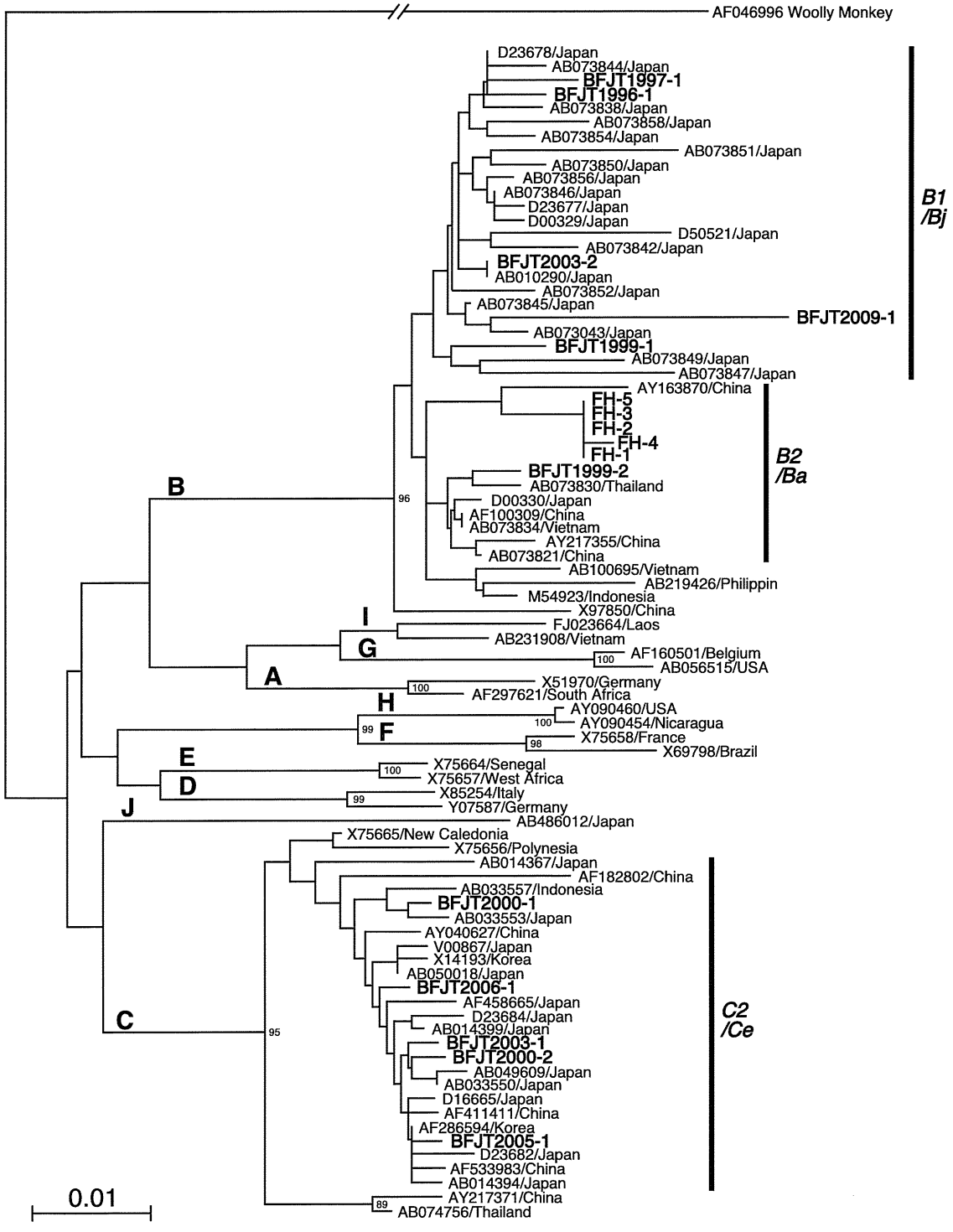


Figure 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial 396-nucleotide sequences in the S region of 84 hepatitis B virus isolates. In addition to the 16 isolates found in this study, which are indicated in bold type for visual clarity, 68 reported isolates of genotypes A–J were included for comparison. Bootstrap values are indicated for the major nodes as a percentage obtained from 1000 resamplings of the data.

1754, 1762/1764, 1862, 1896, and 1899 were found in 17%, 33%, 42%, 8%, 67%, and 25% of the 12 isolates, respectively. Among the subgenotypes, there were differences in the distribution of the mutations: T1754G was found only in subgenotype B1/Bj (4 of 5, 80%), the mutations at nucleotide 1762/1764 were found in subgenotype B2/Ba and C2/Ce (5 of 7, 71%), and G1899A was found only in subgenotype B1/Bj (3 of 5, 60%). Interestingly, an insertion/deletion of a single nucleotide in the precore region (1838insA, 1846del, and 1901insA) was also found only in subgenotype B1/Bj (3 of 5, 60%). The surrounding nucleotide

Table 2. Mutations, Insertions, and Deletions of Hepatitis B Virus Found in the Fulminant Hepatitis Patients

Isolate name	Subgenotype	Nucleotide no.[a]						Frameshift[b]
		1753	1754	1762/1764	1862	1896	1899	
BFJT1996-1	B1/Bj	T	T	A/G	G	G	**A**	**1846del**
BFJT1997-1	B1/Bj	T	**G**	A/G	G	G	G	**1901insA**
BFJT1999-1	B1/Bj	T	**G**	A/G	G	**A**	**A**	...
BFJT2003-2	B1/Bj	T	**G**	A/G	G	**A**	G	**1838insA**
BFJT2009-1	B1/Bj	**Y**	**G**	A/G	G	**A**	**A**	...
BFJT1999-2	B2/Ba	T	T	A/G	G	**A**	G	...
FH-1,2,3,4,5[c]	B2/Ba	T	T	**T/A**	**T**	**A**	G	...
BFJT2000-1	C2/Ce	T	T	**T/A**	G	**A**	G	...
BFJT2000-2	C2/Ce	T	T	**T/A**	G	**A**	G	...
BFJT2003-1	C2/Ce	T	T	A/**A**	G	**A**	G	...
BFJT2005-1	C2/Ce	**G**	T	**T/A**	G	G	G	...
BFJT2006-1	C2/Ce	T	T	A/G	G	G	G	...
Frequency (%)		17	33	42	8	67	25	25

NOTE. 1846del, a single nucleotide deletion at nucleotide 1846; 1901insA, an insertion of A between nucleotide 1900 and 1901; 1838insA, an insertion of A between nucleotide 1837 and 1838.

[a] The nucleotides of mutation are indicated in bold type.

[b] Insertion/deletion that causes a frameshift in the precore protein.

[c] These isolates are indicated as a single strain because of the high identity [23].

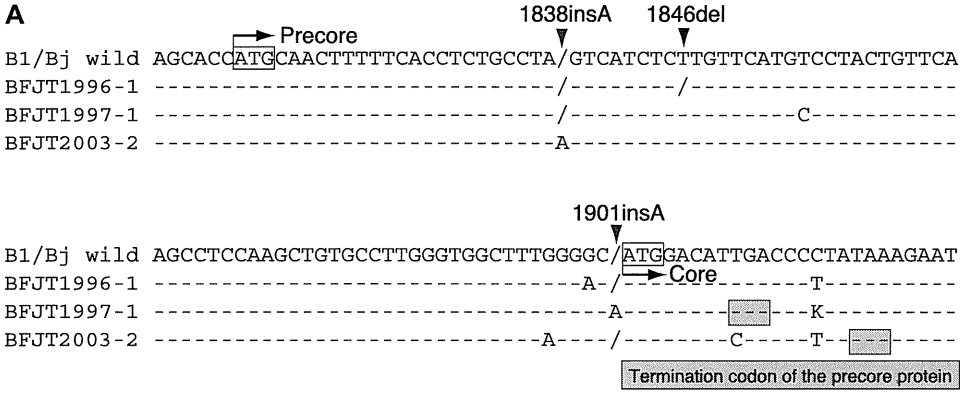
sequences of the single nucleotide insertions/deletions in this study are shown in Figure 2. The insertions in this region make a termination codon at nucleotide 1909 or 1915 in a frame of the precore protein, and the deletion makes a termination codon at nucleotide 1993. Therefore, these single nucleotide insertions/deletions resulted in frameshifts of the precore protein, and they were thought to abrogate HBeAg expression. These precore frameshift mutants were previously found in HBeAg-negative HBV carriers [25, 26]. As for self-limited acute hepatitis B patients, we found that only 1 of 96 (1%) patients had the frameshift mutant (data not shown).

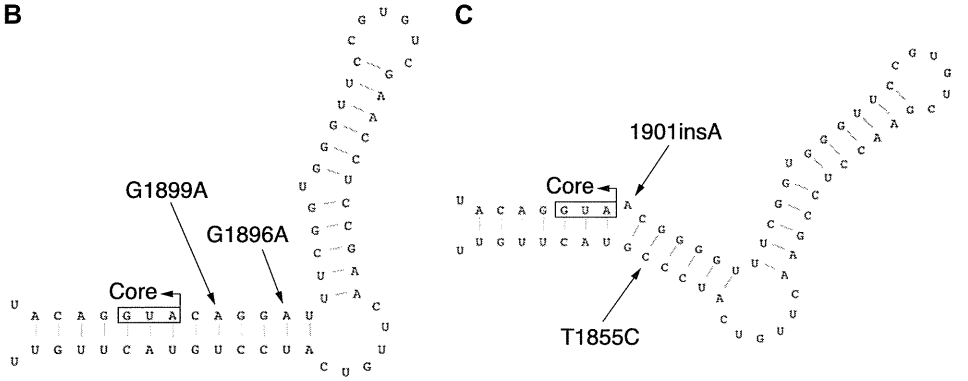
With the aim of clarifying the distribution of the precore frameshift insertion/deletion in the general population, the HBV isolates whose entire sequences were known were retrieved from the Hepatitis Virus Database [27]. In November 2010, a total of 3457 full-length sequences of HBV were registered, and of these, 3391 sequences were proved to belong to genotypes A–I based on a phylogenetic tree analysis. In total, 11 (0.3%) isolates with the precore frameshift were found in genotypes A, B, C, and D isolates (Table 3). Therefore, the frameshift seemed to be rare in general, but can occur in several genotypes other than genotype B.

Of the mutations, insertions, and deletion in the precore region found in the FH patients, G1896A, G1899A, and 1901insA can affect the ϵ signal of HBV. The ϵ signal, which forms a secondary structure of pregenomic RNA, is highly conserved among HBV strains and is essential for the initiation of the encapsidation of pregenomic RNA [28]. Figure 2 depicts the structure of the ϵ signal with G1896A and G1899A, which stabilize the nucleotide pair in the lower stem. The stabilized ϵ signal has an advantage for pregenomic RNA encapsidation [28] and is considered to lead to heightened replication efficiency of HBV. However, 1901insA distorted the secondary structure of the lower stem and seemed to make the ϵ signal wobble (Figure 2). The mutation of T1855C, which might compensate for the instability, was present along with 1901insA in the BFJT1997-1 isolate. The change of the ϵ signal stability was evaluated by calculating the minimum free energy (Figure 2): the lower energy value indicates higher stability of the structure. It was indicated that the ϵ signal with G1896A and/or G1899A had higher stability than the wild type. The structure with 1901insA has lower stability, and it was confirmed that the mutation of T1855C restores the stability, which was still lower than that of the wild type.

Effect of the Precore Frameshift on HBV Replication In Vitro

To confirm whether the frameshift in the precore region of subgenotype B1/Bj HBV has significance in the development of FH, the replication capacity of the HBV clones with the frameshift insertion/deletion (1838insA, 1846del, and 1901insA) was evaluated in vitro using plasmids containing the 1.3-fold HBV genome (Figure 3). As expected, the level of HBsAg in the culture supernatant was almost equal, and the HBeAg level of the clones with the frameshift was significantly lowered to the same level as the clones with G1896A, which is known to abrogate HBeAg. When the amount of HBV DNA in the culture supernatant was assayed, it was revealed that the clones with the frameshift had significantly higher HBV DNA levels than did the wild type. The HBV DNA level of the clone with G1896A ± G1899A was significantly higher, also.





D

Mutation/insertion in the ε signal	Minimum free energy (kcal/mol)
B1/Bj wild type (D00329)	-24.9
G1896A	-25.5
G1899A	-26.1
G1896A+G1899A	-26.7
1901insA	-21.0
1901insA+T1855C	-24.1

Figure 2. *A*, Partial sequences around the precore region of hepatitis B virus (HBV) with frameshift insertion/deletion obtained in this study. The sequence (nucleotides 1808–1925) of a subgenotype B1/Bj wild-type isolate (D00329) is shown on the first line for comparison. The white boxes indicate the initiation codons of the precore/core protein, and the gray boxes indicate the termination codons of the precore protein resulting from the frameshift insertions. *B*, Secondary structure of the ϵ signal of HBV pregenomic RNA with the mutations of both G1896A and G1899A, which are found commonly in fulminant hepatitis patients and hepatitis B e antigen (HBeAg)–negative carriers. *C*, Secondary structure of the ϵ signal with both 1901insA and T1855C found in the BFJT1997-1 isolate. *D*, Stability of the ϵ signal evaluated with minimum free energy, which was calculated using Genetyx Mac. The mutation/insertion was assumed to be present in the B1/Bj wild-type sequence (D00329) and analyzed.

Because the BFJT1997-1 isolate with 1901insA had T1855C in the precore region, the effect of the mutation on HBV replication was evaluated (Figure 3). Interestingly, T1855C increased significantly the HBV DNA level of the clone with 1901insA. The clone with T1855C without 1901insA did not increase the HBV DNA level in comparison with that of the wild type. Therefore, the effect of T1855C was considered to be a restoration of the ϵ signal instability with 1901insA as described above.

The amount of the intracellular replicative intermediates of HBV was evaluated with Southern blot analysis (Figure 3). The result was concordant with that of the HBV DNA level in the culture supernatant. This indicated that the precore frameshift had an effect in the replication cycle before the release step of virion, such as the encapsidation of pregenomic RNA.

Change of the Core Protein Expression Level With the Frameshift Insertion

Although it is considered that the stability of the ϵ signal is necessary for efficient replication [28], the structure of the ϵ signal with 1901insA and T1855C seemed not to be more stable than the wild type as shown in Figure 2. Therefore, another mechanism by which the HBV replication is enhanced was assumed

Table 3. Distribution of Insertions and Deletions That Cause Frameshift of the Precore Protein Among HBV Genotypes A–I, Based on the Isolates Whose Full-length Sequences Were Known

			Subgenotype									
Genotype[a]	A	B	B1	B2	C	D	E	F	G	H	I	Total
No.	427	856	40	659	1191	499	249	77	26	30	36	3391
Insertion	2	1	1	0	1	1	0	0	0	0	0	5
Deletion[b]	1	2	0	2	3	0	0	0	0	0	0	6
Total (%)	3	3	1	2	4	1	0	0	0	0	0	11
	(0.7)	(0.4)	(2.5)	(0.3)	(0.3)	(0.2)	(0)	(0)	(0)	(0)	(0)	(0.3)

NOTE. HBV, hepatitis B virus.

[a] If the recombination of the genome among different genotypes was present, the genotype of HBV was determined by the phylogenetic tree analysis based on the full-length HBV sequences.

[b] The isolates that had deletions including the precore initiation codon were not counted as having the frameshift deletion.

to act. We focused on the change of the Kozak sequence around the initiation codon of the core protein. The Kozak sequence includes the 6-nucleotide sequence just before the initiation codon and 1-nucleotide after that (optimal sequence, GCCA/GCCATGC), and it affects the translation efficiency [29]. Figure 4 shows the altered Kozak sequence of the core protein with G1896A, G1899A, and 1901insA. Whereas G1896A and G1899A make a 1-nucleotide change in the Kozak sequence, 1901insA makes a 3-nucleotide change. A cell-free protein expression system was used to clarify whether the Kozak sequence alteration affects the core protein expression. Western blot analysis of the expressed core protein showed that the Kozak sequence with G1896A or G1899A increased the expression slightly in comparison with the wild-type sequence and, notably, that the Kozak sequence with 1901insA increased the protein level greatly (Figure 4). This increment of the core protein may enhance the replication of HBV particles and may lead to the development of FH.

DISCUSSION

It has been considered that FHB results from the rapid increase of HBV and the vigorous host immune response to HBV-infected hepatocytes [30, 31]. Several mutations found in HBV of FH patients were reported to enhance the HBV replication in vitro [1, 10]. We previously reported that a FH strain caused intracellular retention of HBV, which was thought to be associated with pathogenesis [20]. Here, we described that the single nucleotide insertion/deletion in the precore region leading to a frameshift, which abrogates HBeAg, was found frequently in our FH patients with subgenotype B1/Bj HBV. The frameshift mutants had never been reported in self-limited acute hepatitis patients, whose HBeAg-positive rate is high (56%–84%) [10, 11, 32, 33]. The number of patients in this study was small, but the significance of the frameshift could be confirmed using an in vitro HBV replication system. Although HBV isolates with the frameshift were rarely found in general, patients with acute infection with these isolates may be at risk of developing FH.

This study showed that genotype B HBV was found frequently (69%) in the FHB patients in our hospital in northeast Japan. Recently, the frequency of genotype B in chronic hepatitis B patients in northeast Japan was reported to be higher than that in all Japan (44% vs 14%, respectively) [18]. Although the percentage of genotype B in FHB patients in the area had not reported, this study confirmed that the genotype B percentage was higher. Whereas genotype B HBV frequently leads to FH [9–11], it causes less progressive chronic liver disease than genotype C. This phenomenon was considered to link to earlier HBeAg/Ab seroconversion in the natural course of genotype B compared with that of genotype C [34].

In this study, the precore frameshift was found exclusively in subgenotype B1/Bj strains. The database search showed that the frameshift could occur in several genotypes, at least genotypes A–D, but not frequently (0.3% in total). Interestingly, a previous report by Sugauchi et al showed that 7 of 275 (2.5%) chronically infected patients with subgenotype B1/Bj had the frameshift insertion of 1838insA [35]. Because the number of genotype B1/Bj isolates in that report is larger than that from the database search of full-length HBV sequences, this frequency is more convincing. This may be one of the reasons why subgenotype B1/Bj HBV frequently causes FH.

Previous reports described that HBV with G1896A had a high replication capacity [10], and the present study showed that the precore frameshift insertion/deletion also enhanced the HBV replication to the same level as G1896A. It was reported that p22, the N-terminal-processed p25 precore protein, inhibited the formation of nucleocapsids and regulated the HBV replication [16]. The protein of p22 is further modified at the C-terminal region to secrete p17 HBeAg. HBV with G1896A or the frameshift insertion/deletion in the precore region does not express p25 and the resulting p22. If there is no p22, the nucleocapsids are formed efficiently and the replication of HBV particles can be accelerated. However, this contravenes the general course of HBV carriers, whose seroconversion of HBeAg/HBeAb leads to the reduction of serum HBV DNA

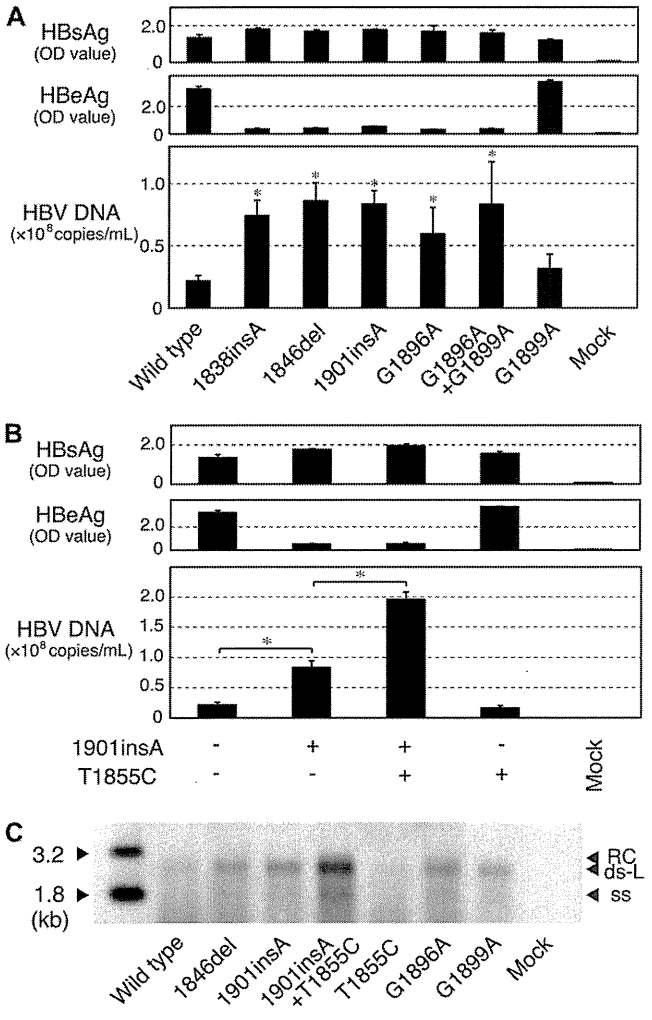


Figure 3. *A*, Level of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and hepatitis B virus (HBV) DNA in the culture supernatant of HepG2 cells that were transfected with several HBV constructs with a frameshift insertion/deletion or mutations in the precore region. *, $P < .05$ in comparison with the wild type. *B*, Level of HBsAg, HBeAg, and HBV DNA in the culture supernatant of HepG2 cells transfected with HBV constructs with 1901insA and/or T1855C. *, $P < .05$. *C*, Representative data of the intracellular replicative intermediates of HBV detected with Southern blot analysis. ds-L, double-stranded linear HBV DNA; OD, optical density; RC, relaxed-circular HBV DNA; ss, single-stranded HBV DNA.

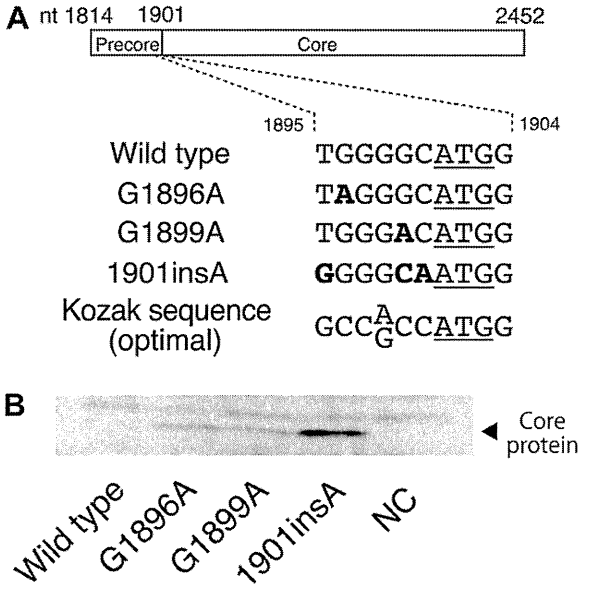


Figure 4. *A*, Schema of the Kozak sequence around the initiation codon of the core protein. The changed nucleotides in the Kozak sequene, which were found in hepatitis B virus (HBV) with G1896A, G1899A, or 1901insA, are shown in bold type. *B*, Results of Western blot analysis of the expressed HBV core protein in a cell-free protein expression system. NC, negative control.

[17]. The discrepancy may be due to the adaptive immune response in HBV carriers. Under the suppression by cytotoxic T lymphocytes, HBeAg-negative HBV clones, which have an advantage in the replication cycle, may be barely persistent in the late phase of HBV infection.

The ϵ signal of HBV pregenomic RNA is recognized by HBV polymerase, and both of them are encapsidated into the core particle [36]. Stability of the ϵ signal favors replication [28] and, therefore, G1896A and G1899A may easily occur in the natural course of HBV infection. However, 1901insA, which was found in an FH patient, degrades the stability. It was compensated by a distinct mutation of T1855C, but seemed not to be so stable based on the secondary structure of the ϵ signal. This in vitro study revealed that the enhancement of HBV replication by the novel insertion of 1901insA resulted from the change of the Kozak sequence of the core protein. It was also interesting that G1896A and G1899A increased the core protein expression level slightly. As for the Kozak sequence in HBV, the sequence just upstream of the precore initiation codon was described previously [37]: it affects the expression of HBeAg, and associates with the seroconversion of HBeAg/Ab. There is a possibility that the Kozak sequence of other HBV proteins such as polymerase, HBsAg, and X protein may alter the HBV replication capacity or the disease outcome.

In conclusion, the frameshift insertion/deletion in the precore region was found frequently in subgenotype B1/Bj HBV from FH patients in northeast Japan. The frameshift was shown to enhance the HBV replication in vitro and, in particular, the insertion of 1901insA heightened the replication capacity via the novel mechanism of the changed Kozak sequence of the core protein. Therefore, the precore frameshift may have significance in the development of FH.

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肝硬変に出現する血漿アミノ酸不均衡が免疫機構に与える影響

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索引用語：肝硬変，分岐鎖アミノ酸，栄養免疫

1 はじめに

厚生労働省の報告によると平成21年の死亡原因において肝疾患は15,969人，肝臓癌は32,725人と肝臓関連死の割合は極めて高く，これらの患者の大部分に肝硬変が存在する．肝硬変では，糖・蛋白・アミノ酸・脂質・ビタミン・ミネラルなど極めて多岐にわたる栄養代謝障害が出現する．特に血漿中のアミノ酸不均衡に関しては分岐鎖アミノ酸(BCAA)が低下し芳香族アミノ酸(AAA)が増加しFischer比が低下することが古くから知られている[1,2]．進行した肝硬変では肝癌・肝不全だけでなく肺炎・特発性細菌性腹膜炎(SBP)などの感染症が予後を左右する．これは，肝硬変患者は易感染性宿主であり免疫異常が存在することを示唆する．

近年の基礎的な研究報告によるとアミノ酸はただ単に細胞骨格やさまざまな代謝経路の基質となるだけでなく，インスリンとともにmTORを介した栄養感受性シグナル経路に作用し，細胞機能を調節することが解明されてきている[3~5]．さらに，免疫に関してもmTORシグナルが抗原提示細胞の機能に関与することが報告されてきている[6,7]．

本稿では免疫に影響を与える代表的な遊離アミノ酸について過去の文献を基に解説するとともに肝硬変に出現する血漿中アミノ酸不均衡が免疫細胞にいかなる影響を与えるのかを考察する．

2 免疫細胞に影響を与える遊離アミノ酸

1. グルタミン(L-Glutamine: L-Glu)

非必須アミノ酸であるグルタミンは以前よりリンパ球を含めた細胞増殖に重要であることが知られ[8]，細胞培養の際に培養液に添加される．*In vitro*において細胞外のグルタミン濃度を高めるとT-cellの増殖・IL-2産生，B-cellの形質細胞への分化も亢進する[9]．さらにリンパ球の増殖だけでなく単球やマクロファージの機能にも関与しており，培地

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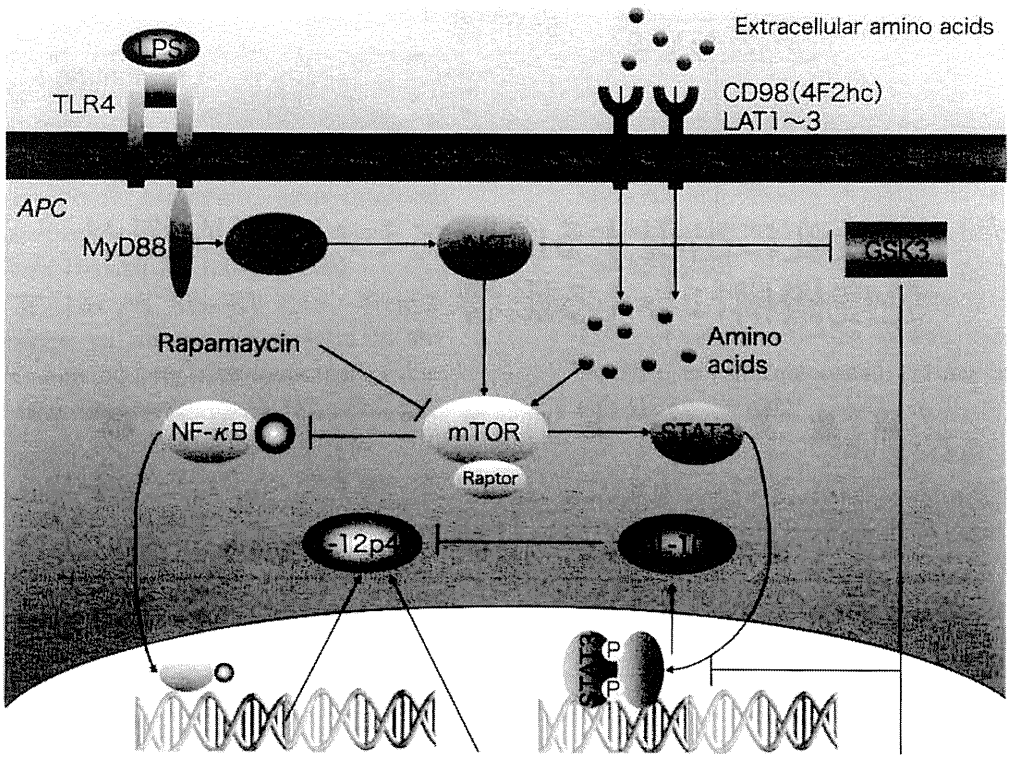

図1　抗原提示細胞におけるmTORシグナルとサイトカイン産生の関係

中のグルタミンの除去により細胞内ATP産生低下に伴う機能低下(MHC class Ⅱ発現低下，貪食能低下，TNF-alpha IL-8の産生低下)が報告されている[9]．最近ではグルタミンとロイシンの双方向性の移動がアミノ酸トランスポーターのSLC7A5/SLC3A2で行われ，mTORシグナルに関与しているとの報告がなされた[10]．肝硬変患者においては，肝硬変の進行とともに血漿中のグルタミン濃度は上昇する傾向がある．これは，肝硬変ではアンモニア代謝のためにL-グルタミン酸→L-グルタミンへの変換が亢進していることによる．

2. 分岐鎖アミノ酸(BCAA: L-Valine: L-Val, L-Leucine: L-Leu, L-Isoleucine: L-Ile)

分岐鎖アミノ酸のL-ロイシンがmTORシグナルを調節することが知られ[4,5]盛んに研究が進められている．免疫に関してはmTOR阻害剤であるラパマイシンが樹状細胞のIL-10産生を抑制しIL-12の産生を亢進させ(図1)Th1 / Th2バランスを調節すること[6]，当科では分岐鎖アミノ酸の中でL-バリンが樹状細胞の成熟化に関与していることを近年報告した[11]．分岐鎖アミノ酸の臨床的効果に関しては，肝硬変患者の低下した好中球の貪食能やナチュラルキラー細胞(NK)の活性化が改善されること[12]，さらに樹状細胞機能も改善されること[13]が報告されている．

3. トリプトファン(L-Tryptophan: L-Trp)

トリプトファンをキヌレニンに代謝する酵素Indoleamine 2,3-dioxygenase (IDO)はTリンパ球の増殖を抑制し免疫寛容の成立に関与しているとの報告がある[14]．逆に細胞外の過剰なトリプトファン濃度はIDOを介した抑制を阻害することが知られている[15]．東谷らは慢性C型肝炎患者では樹状細胞のIDO活性がLPS+IFN-γ刺激時に亢進しており制御性T細胞の誘導に関与することを報告し

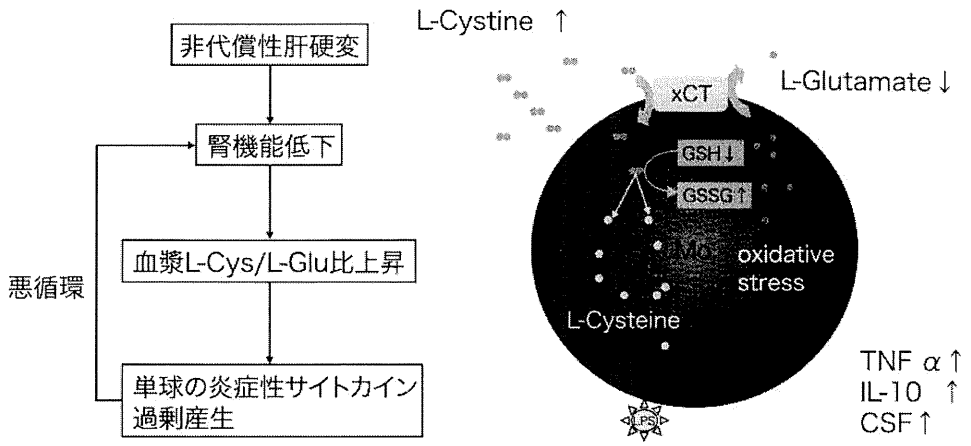

図2　非代償性肝硬変におけるL-シスチン／L-グルタミン酸不均衡が単球に与える影響

た[16].

4. アルギニン(L-Arginine：L-Arg)

アルギニンは腎臓において腸管由来のシトルリンから内因性に産生される非必須アミノ酸である．活性化マクロファージの一酸化窒素(NO)は，誘導型NO合成酵素(iNOS)によりアルギニンから産生され多くの病原体に対する宿主防衛に必須であるので，アルギニンは感染抵抗性の重要な決定因子である[17].

さらに，アルギニンが枯渇するとTLR4シグナル下流のTPL-2の脱リン酸化が促進しMAPKパスウェイを抑制しTNF-αの産生が抑制されることが知られている[18]．アルギニンが肝硬変患者の免疫に直接与える影響に関しては不明であるが，肝硬変患者，特にSBP患者では腹水中のマクロファージのNOSが亢進していることが古くから知られているため，アルギニンの需要は亢進しているものと考えられる．

5. シスチン・グルタミン酸(L-Cystine: L-Cys / L-Glutamate: L-Glu)

古くからL-シスチンとL-グルタミン酸は哺乳類細胞において交換輸送されることが知られていたが，近年その交換輸送体xCTがSato Hらによりクローニングされた[19]．L-シスチンは細胞内に入るとL-システインに還元されグルタチオンの産生に使用される．免疫細胞に関しては，xCTは単球系細胞に発現しているがリンパ球には発現しておらず，さらに抗原提示細胞が放出するL-システインはリンパ球の増殖を調節することが報告されている[20,21]．一方肝硬変患者においては，肝硬変が進行すると血漿中のL-シスチンは増加し，L-グルタミン酸は低下する．特にL-シスチンは腎機能障害が出現すると著明に増加し，LPS刺激を受けた単球からのTNF-αの産生を増加させることが当科の研究で明らかとなった．これらの結果から，L-シスチンが非代償性肝硬変の腎機能に悪循環を与えている可能性を示唆している(図2)．

免疫細胞に作用する代表的な遊離アミノ酸を取りあげたが，他のいくつかのアミノ酸に関しても免疫に作用する報告があり，今後さらなる研究が進められると考える．

3 肝硬変で出現するアミノ酸不均衡と免疫へ

肝硬変患者の実際の免疫状態に関しては，血漿中のTNF-alpha, IL-6, IL-1βなどの各種炎症性サイトカインが高いことが古くから

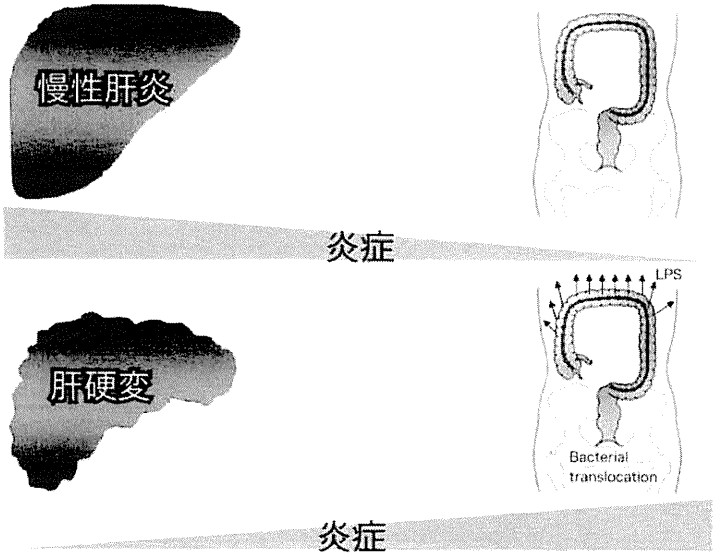

図3　慢性肝炎と肝硬変の炎症場所の違い

表　慢性肝炎・肝硬変患者の血漿中アミノ酸濃度

	CH (n=33)	Child A (n=81)	Child B (n=57)	Child C (n=35)
Glycine	242	216	242	318
L-Alanine	393	320	311	368
L-Serine	130	133	143	178
L-Threonine	144	143	149	178
L-Cystine	56	60	68	72
L-Methionine	31	34	52	70
L-Glutamine	596	607	662	763
L-Asparagine	56	56	64	80
L-Glutamic acid	78	66	55	52
L-Aspartic acid	4	3	4	5
L-Valine	247	222	190	184
L-Leucine	142	122	107	105
L-Isoleucine	73	63	56	57
L-Phenylalanine	72	78	98	112
L-Tyrosine	85	98	124	164
L-Tryptophan	53	50	47	46
L-Lysine	222	202	193	198
L-Arginine	76	83	88	106
L-Histidine	89	83	80	96
L-Proline	178	156	165	224
Fischer' s ratio	3.04	2.39	1.70	1.29

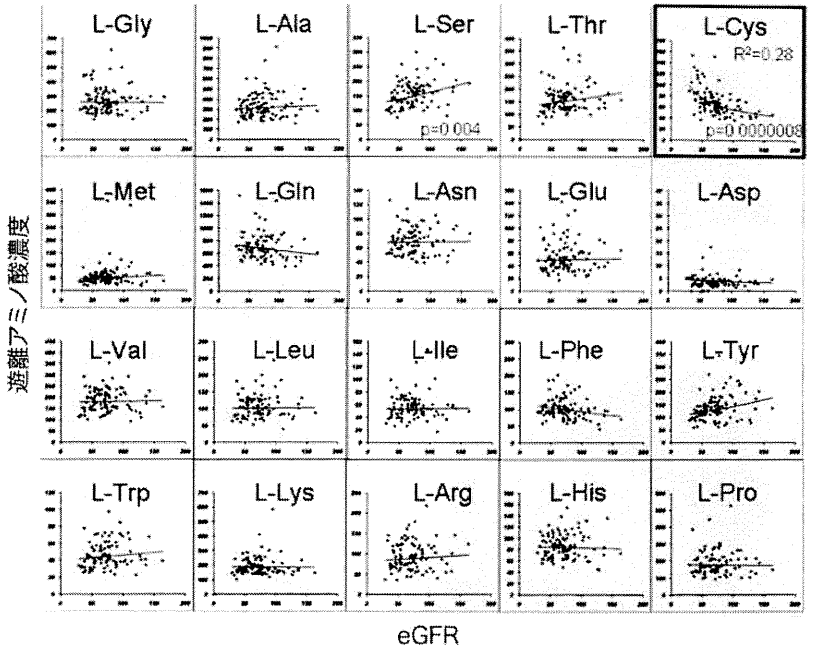

図4　Child-Pugh gradeB orC患者の腎機能と血漿中遊離アミノ酸の関係

知られている[22,23]が，慢性肝炎とは異なり肝硬変では肝臓内での炎症よりもむしろ肝臓外の炎症(細菌感染)による影響を考えなければならない(図3)．マウスを用いた実験では肝硬変では腸管からの細菌刺激(Bacterial translocation)が頻回に起こり，各種免疫細胞の活性状態が持続しているとの報告がある[24～27]．

健常人→慢性肝炎→肝硬変と病状の進行により血漿中のアミノ酸不均衡が出現することは先に述べたが，肝硬変ではChild-Pugh (C-P) grade BやCでその不均衡は著明になる(表)．これは，非代償性肝硬変になると肝臓だけでなく骨格筋や腎臓でのアミノ酸代謝に異常が出現するためである．しかしC-P gradeにはこれらのパラメータは含まれない．C-P grade BまたはCに関して腎機能(eGFR)と各種アミノ酸を比較統計するとC-P gradeではマスクされたアミノ酸異常が明らかとなる(図4)．

これら個々のアミノ酸の変動が免疫に対して良い影響を与えているのか悪影響を与えているのかは依然として不明な点が多いが，BCAAなどの低下しているアミノ酸に関しては，前述のとおり免疫細胞の全アミノ酸需要が亢進していることを考慮すると補充することが良いと考える．一方で増加してくるアミノ酸に関しては，肝硬変の免疫反応に対して悪影響を与えている可能性がある．また，トリプトファンなどの一部のアミノ酸に関しては毒性がいわれており，わずかな変動が免疫に大きな影響を与える可能性がある．

4　今後の展開と問題点

肝硬変に出現する血漿中のアミノ酸不均衡が免疫機構へ影響を与えていることは確実である．細胞培養の際にD-MEM, RPMI 1640などの市販されている培地は各種アミノ酸濃度が生体内の濃度よりもはるかに高濃度であるためこの中でアミノ酸の機能解析はできない．さらに，希釈したものを用いたとしても各種アミノ酸は交換輸送されることが多く，

HCM(healthy control medium)
健常人の血漿アミノ酸濃度に一致

ACM(advanced cirrhotic medium)
非代償性肝硬変患者(Child-Pugh grade BまたはC)の血漿アミノ酸濃度に一致

(細胞科学研究所　製造)

	CCM	HCM	ACM
Glycine	400	225	280
L-Alanine	400	391	307
L-Serine	400	119	151
L-Threonine	800	142	138
L-Cystine 2HCl	200	38	67
L-Methionine	200	29	75
L-Glutamine	4000	564	689
L-Asparagine	400	51	64
L-Glutamic Acid	400	42	53
L-Aspartic Acid	400	3	4
L-Valine	800	249	175
L-Leucine	800	132	100
L-Isoleucine	800	76	53
L-Phenylalanine	400	63	99
L-Tyrosine	400	65	133
L-Tryptophan	80	62	45
L-Lysine-HCl	800	183	184
L-Arginine-HCl	400	78	92
L-Histidine HCl-H2O	200	83	85
L-Proline	400	204	176
Fischer's ratio	3.00	3.57	1.42

nmol/mL

Kakazu E, Ueno Y, et al. Hepatology 2009

図5　当科で開発したアミノ酸研究用無血清培地

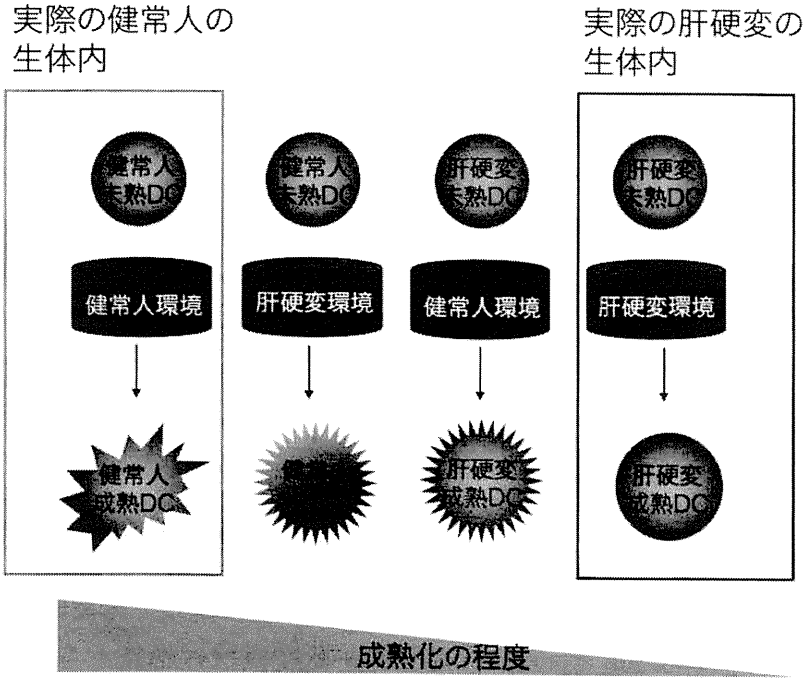

図6　非代償性肝硬変では樹状細胞自体の成熟化が抑制されているだけでなく，血漿中のアミノ酸不均衡が成熟化を抑制する

個々のアミノ酸バランスが重要であるため厳密に生体内の環境を反映しているとはいえない．これらの問題点を解決するために当科では実際の健常人や肝硬変患者の血漿中アミノ酸濃度に一致した無血清培地を開発した(図5)．この培地を用いることで初めて個々のア

ミノ酸を培地に添加しその影響を解析できるようになり，非代償性肝硬変では健常人と比較して樹状細胞自体の成熟化が抑制されているだけでなく細胞外のアミノ酸環境が樹状細胞の成熟化を抑制することを明らかにした(図6)[13]．しかしそれでも生体内においては食事，体内水分量，さらにその局在(末梢血，門脈血，各種臓器・リンパ節など)により細胞外のアミノ酸濃度は異なるためその点を考慮しなければならない．

5 おわりに

前向き研究によりBCAA製剤は，肝硬変患者に対して肝不全イベントの低下，QOLの改善，さらに発癌リスクを低下させる効果を持つことが報告され[28,29]，臨床試験でのBCAAの有効性が証明されている．また近年，本多らにより肝細胞自体のインターフェロンシグナルを改善させる効果も報告された[30]．慢性C型肝炎・肝硬変患者の高齢化により，副作用の少ない栄養療法の重要性が増していくことは明白である．遊離アミノ酸の免疫細胞に与える影響がより詳細に解析されれば，①感染症予防，②発癌抑制，③C型肝硬変患者のインターフェロン奏効率向上につながる可能性がある．

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Lymphotropic hepatitis C virus has an interferon-resistant phenotype

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SUMMARY. Hepatitis C virus (HCV) infects and associates with B cells, leading to abnormal B-cell activation and development of lymphoproliferative and autoimmune disorders. This immune perturbation may in turn be associated with the resistance of HCV against the host immune system. The objective of this study was to analyse the effects of HCV infection of B cells on the efficacy of interferon (IFN)-based therapy. The study enrolled 102 patients with chronic hepatitis C who were treated with pegylated IFN plus ribavirin. HCV RNA titres in B cells were compared in patients with rapid viral responder (RVR) *vs* non-RVR, sustained viral responder (SVR) *vs* non-SVR and null viral responder (NVR) *vs* VR. The levels of HCV RNA in B cells were significantly higher in non-RVR, non-SVR and NVR groups. Association between the therapy outcome and the positive B-cell HCV RNA was also investigated in relation to other known viral and host factors. Multivariable analyses showed that the positive B-cell HCV RNA and the minor single-nucleotide polymorphism near the IL28B gene (rs8099917) were independent factors associated with NVR in patients infected with HCV genotype 1. When these two factors were combined, the sensitivity, specificity, positive and negative predictive values for NVR were 92.3%, 98.2%, 92.3% and 98.2%, respectively. Genotype 1 and the presence of one or no mutations in the IFN-sensitivity determining region were associated with higher levels of B-cell HCV RNA. B-cell-tropic HCV appears to have an IFN-resistant phenotype. B-cell HCV RNA positivity is a predictive factor for resistance to IFN-based therapy.

Keywords: B-cell disorders, B-cell tropism, hepatitis C virus, interferon resistance, lymphoproliferative disorders.

INTRODUCTION

Hepatitis C virus (HCV) infects 200 million people worldwide, causing chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [1,2]. In addition, HCV infection causes proliferative disorders of B cells, such as mixed cryoglobulinemia [3–5] and B-cell non-Hodgkin's lymphoma [6]. Even when no clinical extrahepatic manifestations are observed, 74% of patients infected with HCV present 'biological' extrahepatic manifestations, such as cryoglobulinemia, high levels of rheumatoid factor, hypocomplementemia and clonal expansion of B cells [7]. Molecular mechanisms of these B-cell abnormalities remain to be clarified. Replication of HCV is observed in the peripheral blood mononuclear cells (PBMCs), especially in B cells of patients with chronic hepatitis C (CH-C) [8–10]. In our previous report, HCV RNA was detected in B cells from 64.0% of CH-C patients and negative-strand HCV RNA in 5.3% of these patients [7]. The presence of lymphoproliferative disorders (LPDs) is associated with HCV RNA detection in B cells. Furthermore, the IFN responses of peripheral B cells from CH-C patients have been reported to be impaired [11]. Taken together, these results indicate that B-cell-tropic HCV exists and may play an important role in the immunological dysfunction of B cells.

As the discovery of HCV, rapid progress has been made in the development of antiviral therapies against HCV infection. The current standard treatment for CH-C, a combination of pegylated interferon (PEG-IFN) and ribavirin for 24 or 48 weeks, has improved clinical responses [12,13]. However, even with this regimen, half of CH-C patients do not achieve sustained clearance of HCV. The likelihood of the response varies greatly, depending on both host and viral factors. Viral factors associated with resistance to interferon (IFN)-based therapy include HCV genotype 1, high viral

Abbreviations: CH-C, chronic hepatitis C; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; LPD, lymphoproliferative disorders; NPV, negative predictive value; NVR, null viral responder; PBMCs, peripheral blood mononuclear cells; PEG-IFN, pegylated interferon; PPV, positive predictive value; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder; VR, viral responder.

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loads and reduced quasispecies diversity in the IFN-sensitivity determining region (ISDR) of NS5A [14], as well as mutations at the 70th and 91st amino acids of the HCV core region [15]. Host factors include age, race, gender, degree of hepatic fibrosis, insulin resistance [16] and interleukin (IL) 28B genotype [17]. Although many of these factors are used for prediction of the response to IFN therapy, it is currently impossible to determine who will attain viral eradication from hepatocytes. Furthermore, examination of all the markers increases medical expenses.

HCV replicates in lymphoid cells at low levels, but they may serve as an extrahepatic reservoir; this is implicated in the recurrence and persistence of HCV infection in immunosuppressed individuals [18]. In addition, low amounts of positive- and negative-strand HCV RNAs are still detected in lymphocytes of sustained viral response (SVR) patients with CH-C after the virus eradication from hepatocytes by IFN-based therapy [19,20]. HCV in lymphoid cells may represent a subpopulation resistant to innate immunity and/or a source of B-cell dysfunction.

The primary purpose of this study was to test the hypothesis that HCV associated with B cells confers resistance to IFN-based therapy and serves as a useful predictive parameter for therapy outcome. The study also evaluated other viral and host factors in conjunction with B-cell HCV RNA as predictive markers for the efficacy of antiviral therapy.

METHODS

Subjects

This study enrolled 102 patients with CH-C who were treated with PEG-IFN plus ribavirin therapy for 24, 48 or 72 weeks (Showa University Hospital, 2005–2010). Diagnosis of HCV infection was based on the detection of anti-HCV antibody and HCV RNA in the serum prior to the initiation of therapy. Clinical characteristics of the HCV-infected patients are shown in Table 1. Missense mutations in the ISDR and in codons 70 and 91 of the core region were analysed in patients infected with HCV genotype 1 by direct sequencing [14,15]. Liver biopsies were performed in 92 patients before the start of therapy; fibrosis was staged from F0 to F4 according to the scheme of Desmet *et al.* [21]. The study protocol was approved by the Ethics Committee of Showa University School of Medicine, Tokyo, Japan. Informed written consent was obtained from each participant and the study followed the ethical guidelines of the 1975 Declaration of Helsinki.

Isolation of B cells

B cells were isolated using an auto-MACS™ Pro Separator ver.2.0.0 (Miltenyi Biotec K.K., Bergisch Gladbach, Germany). Briefly, PBMCs were obtained from whole blood (30 mL) by centrifugation. Non-B cells were labelled by a cocktail of specific antibodies that were conjugated to biotin, and the mixture was adsorbed with the MACS microbeads to capture the biotin-labelled cells. The cell–microbead mixture was then passed through the auto-MACS magnetic column, and B cells (the flow-through) were collected.

Table 1 Clinical characteristics of HCV-infected patients (n = 102)

Age (years)	54.1 ± 11.4
Male/Female	53/49
ALT (IU/L)	73.8 ± 74.4
Platelets ($\times 10^4/mm^3$)	18.4 ± 6.5
Fibrosis (F0/F1/F2/F3/F4)	1/38/38/9/6
Outcome of IFN therapy (RVR/SVR/NVR)	46 (45%)/60 (59%)/ 15 (15%)
IL28B SNPs (T/T *vs* T/G, G/G)	76/99 (77%) *vs* 23/99 (23%)
Cryoglobulinemia	24/98 (24%)
IgG (mg/dl)	1712 ± 504
IgA (mg/dl)	253 ± 118
IgM (mg/dl)	119 ± 64
Rheumathoid factor (>10 IU/mL)	39/99 (39%)
C3 (<86 mg/dL)	16/99 (16%)
C4 (<10 mg/dL)	4/99 (4%)
CH50 (<20 U/mL)	59/97 (58%)
Presence of B cell clonality	9/71 (12.7%)
HCV genotype (1/2)	77 (75.5%)/25 (24.5%)
Log HCV RNA in serum (log/mL)	5.9 ± 0.82
Positive of HCV RNA in B cells (>10 log/100 ng)	58/102 (57%)

HCV, hepatitis C virus; NVR, null viral responder; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder
Continuous variables are presented as mean ± standard error.

Quantitation of HCV RNA in B cells

Total RNA from each cellular compartment was extracted using the AllPrep® DNA/RNA/Protein Mini kit (Qiagen, Duesseldorf, Germany). HCV RNA levels were determined in 100 ng of each RNA sample by real-time RT-PCR using the primers described previously [22]; this assay has a detection range over 1.0–8.0 log copies. Samples were scored as positive for HCV RNA when titres exceeded 1.0 log copies/100 ng; this threshold excluded contamination of lymphoid cells with serum HCV RNA [7].

IL28B SNP genotyping assay

In 99 patients, the genotype of an IL28B-proximal single-nucleotide polymorphism (SNP) (rs8099917) was determined by real-time PCR. For each patient, genomic DNA (10–100 ng) was purified (Qiagen) and amplified using the

SNP Genotyping Assay specific for rs8099917 (Applied Biosystems, Foster City, CA, USA) according to the TaqMan® GTXpress™ Master Mix Protocol (Applied Biosystems).

Statistical analysis

The mean of continuous variables, with and without normal distribution, was compared by Student's *t* test or by the Wilcoxon test, respectively. Comparison of discontinuous variables was performed by the chi-squared test or Fisher's exact test. A *P* value of <0.05 was considered to be statistically significant. Values with normal distributions were expressed as the mean ± standard error (SE). For variables that were not distributed normally, data were transformed into log values as required. To examine the relation between patient parameters and the outcome of therapy, candidate independent variables were analysed by the Wald test of logistic regression modelling via multivariable analysis. All statistical analyses were performed using JMP ver. 9 software (SAS Institute, Cary, NC, USA).

RESULTS

The levels of HCV RNA in B cells and IFN treatment response

HCV RNA titres in B cells were compared in patients with rapid viral responder (RVR) *vs* non-RVR (Fig. 1a), SVR *vs* non-SVR (Fig. 1b) and null viral responder (NVR) *vs* VR (non-NVR) (Fig. 1c) using univariable analysis. The levels of HCV RNA in B cells were significantly different in all three comparisons ($P = 0.0001$, 0.0012 and 0.0020, respectively). The results suggest that patients whose B cells have less HCV RNA are more sensitive to IFN-based therapy. Mean HCV RNA titres in B cells of NVR patients showed the highest titre among the three groups (RVR: 1.0 ± 0.2, SVR: 1.2 ± 0.2 and NVR: 2.9 ± 0.4 log copies/100 ng RNA). The fraction of patients scoring positive for the presence of HCV RNA in B cells was also significantly different in each comparison: [RVR: 18/46 (39.1%) *vs* non-RVR: 40/56 (71.4%), $P = 0.0010$], [SVR: 26/60 (43.3%) *vs* non-SVR: 32/42 (76.2%), $P = 0.0010$] and [NVR: 14/15 (93.3%) *vs* VR: 44/87 (50.6%), $P = 0.0016$].

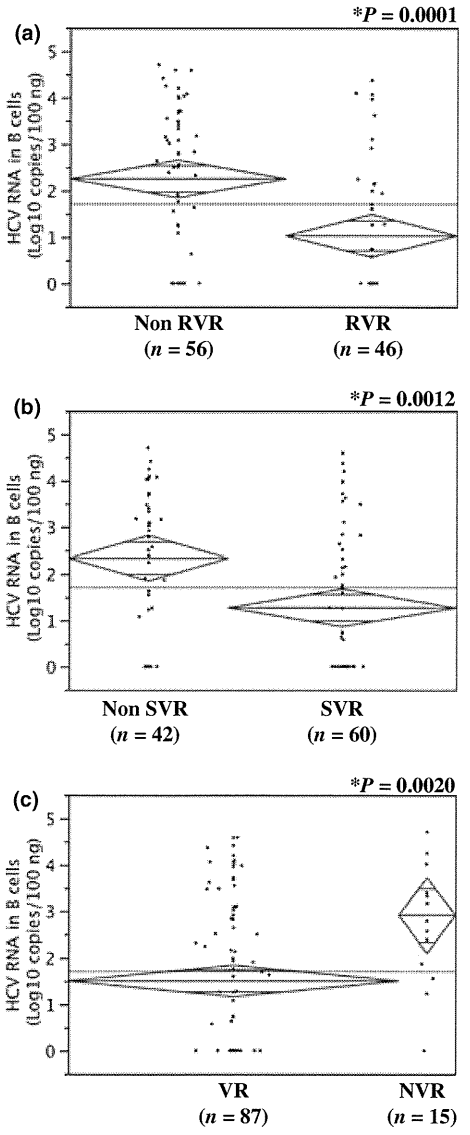

Fig. 1 Comparison of hepatitis C virus (HCV) RNA titres in B cells of patients classified by viral response status: (a) Rapid viral responder (RVR) *vs* non-RVR; (b) sustained viral responder (SVR) *vs* non-SVR; and (c) null viral responder (NVR) *vs* viral responder (VR). A horizontal grey line shows mean of all samples from both groups. Two grey diamonds indicate the averages and 95% confidence interval of each group. Statistical significance was determined by Student's *t* test.

HCV RNA in B cells as a predictive factor for viral response

We next analysed the factors associated with response (RVR, SVR or NVR) to IFN-based therapy. We used the presence of HCV RNA in B cells as a marker of LPD because it is associated with the presence of LPD [7]. Homozygosity for the major allele (T/T) of the IL28B SNP (rs8099917), HCV genotype 2 and low serum HCV RNA levels were significantly associated with RVR by univariable analysis (Table 2a), while the presence of HCV RNA in B cells was associated with non-RVR. When the independence of these factors was assessed by multivariable analysis using a multiple logistic regression model, homozygosity for the major allele (T/T) of the IL28B SNP and lower levels of HCV RNA in sera (<6 log copies/mL) were shown to be independent factors associated with RVR to IFN-based therapy.

In the comparisons of SVR with non-SVR, lower age, higher platelet number, fibrosis stage 1 or 2, the T/T allele of

Table 2 Univariable and multivariable analysis for RVR (a), SVR (b) and NVR (c)

(a) RVR

	Response for therapy		
Univariable analysis	RVR (n = 46)	Non-RVR (n = 56)	P value
Age (years)	52.6 ± 12.0	55.5 ± 10.7	NS
Men	27/46 (59%)	26/56 (46%)	NS
ALT (IU/L)	78.4 ± 68.9	70.0 ± 79.1	NS
Platelets ($\times 10^4/mm^3$)	19.2 ± 6.5	17.8 ± 6.4	NS
Fibrosis stages 1 or 2	36/39 (92%)	41/53 (77%)	NS
IL28B SNPs rs8099917 major allele (T/T)	**39/43 (91%)**	**37/56 (66%)**	**0.0040**
HCV genotype 2	**18/46 (39%)**	**7/56 (13%)**	**0.0019**
Log HCV RNA in serum (log/mL)	**5.45 ± 0.95**	**6.27 ± 0.43**	**<0.0001**
Presence of HCV RNA in B cells (>10 log/100 ng)	**18/46 (39%)**	**40/56 (71%)**	**0.0010**
Multivariable analysis	Odds ratio	95% CI	
IL28B SNP rs8099917 major allele (T/T)	**2.42**	**1.28–5.15**	**0.0116**
HCV genotype 2	1.53	0.84–2.85	NS
HCV RNA in serum <6.0 log/mL	**2.51**	**1.51–4.36**	**0.0006**
Absence of HCV RNA in B cells (<10 log copies/100 ng)	1.38	0.81–2.34	NS

(b) SVR

	Response for therapy		
Univariable analysis	SVR (n = 60)	Non-SVR (n = 42)	P value
Age (years)	**52.1 ± 11.6**	**57.1 ± 10.4**	**0.0247**
Men	32/60 (53%)	21/42 (50%)	NS
ALT (IU/L)	78.1 ± 70.8	67.2 ± 79.7	NS
Platelets ($\times 10^4/mm^3$)	**20.3 ± 7.0**	**15.7 ± 4.6**	**0.0003**
Fibrosis stages 1 or 2	**47/52 (90%)**	**30/40 (75%)**	**0.0477**
IL28B SNPs rs8099917 major allele (T/T)	**52/57 (91%)**	**24/42 (57%)**	**<0.0001**
HCV genotype 2	**21/60 (35%)**	**4/42 (10%)**	**0.0032**
Log HCV RNA in serum (log/mL)	**5.64 ± 0.94**	**6.27 ± 0.35**	**<0.0001**
Presence of HCV RNA in B cells (>10 log/100 ng)	**26/60 (43%)**	**32/42 (76%)**	**0.0010**
Multivariable analysis	Odds ratio	95% CI	
Age <50	1.39	0.77–2.57	NS
Platelets >17 × $10^4/mm^3$	**2.16**	**1.19–4.05**	**0.0139**
Fibrosis stage 1 or 2	1.22	0.56–2.77	NS
IL28B SNP rs8099917 major allele (T/T)	**3.47**	**1.76–7.84**	**0.0009**
HCV genotype 2	1.51	0.70–3.55	NS
HCV RNA in serum <6.0 log/mL	**2.58**	**1.33–5.63**	**0.0085**
Absence of HCV RNA in B cells (<10 log copies/100 ng)	1.66	0.88–3.24	NS

(c) NVR

	Response for therapy		
Univariable analysis	NVR (n = 15)	VR (n = 87)	P value
Age (years)	54.0 ± 2.9	54.2 ± 1.2	NS
Men	10/15 (67%)	43/87 (49%)	NS
ALT (IU/L)	90.5 ± 19.2	71.0 ± 8.0	NS
Platelets ($\times 10^4/mm^3$)	15.5 ± 1.7	19.0 ± 0.7	NS

Table 2 Continued

(c) NVR

Univariable analysis	Response for therapy NVR (n = 15)	VR (n = 87)	P value
Fibrosis stages 1 or 2	4/14 (29%)	11/78 (14%)	NS
IL28B SNPs minor alleles (T/G or G/G)	**12/15 (80%)**	**11/84 (13%)**	**<0.0001**
HCV genotype 1	**15/15 (100%)**	**25/87 (29%)**	**0.0169**
Log HCV RNA in serum (log/mL)	**6.33 ± 0.21**	**5.83 ± 0.09**	**0.0279**
Presence of HCV RNA in B cells (>10 log/100 ng)	**14/15 (93%)**	**44/87 (51%)**	**0.0020**
Multivariable analysis	**Odds ratio**	**95% CI**	
IL28B SNP rs8099917 minor allele (T/G or G/G)	**8.51**	**3.52–27.45**	**<0.0001**
HCV RNA in serum >6.0 log/mL	1.54	0.51–4.85	NS
Presence of HCV RNA in B cells (>10 log copies/100 ng)	**4.80**	**1.57–24.85**	**0.0179**

HCV, hepatitis C virus; NVR, null viral responder; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder
Continuous variables are presented as mean ± standard error.
Bold text indicates statistically significant associations ($P < 0.05$). NS: not significant.

the IL28B SNP, HCV genotype 2 and lower serum levels of HCV RNA were significantly associated with SVR by univariable analysis (Table 2b). Higher platelet counts ($>17 \times 10^4/mm^3$), the T/T allele of the IL28B SNP and lower serum levels of HCV RNA (<6.0 log/mL) were independent factors for SVR by multivariate analysis.

In comparisons of NVR with VR, homozygosity (G/G) or heterozygosity (T/G) for the minor allele of the IL28B SNP, HCV genotype 1, higher serum levels of HCV RNA and the presence of HCV RNA in B cells were significantly associated with NVR by univariable analysis (Table 2c). As none of the NVR patients were infected with HCV genotype 2, multivariable analysis was performed using the 73 samples of HCV genotype 1-infected patients to determine the independent factors associated with NVR. Presence of minor alleles (G/G or T/G) of the IL28B SNP and the presence of HCV RNA in B cells were both found to be independent factors associated with NVR in patients infected with HCV genotype 1.

Stratified analysis of outcome for IFN-based therapy

To assess the utility of HCV RNA in B cells as a predictive factor for the outcome of IFN-based therapy, we repeated the analysis on stratified groups. When the patients were stratified by HCV genotype or by IL28B SNP genotype, the rates of RVR and SVR were higher among cases scoring negative for HCV RNA in B cells (Table 3). In HCV genotype 1-infected patients possessing a minor allele of the IL28B SNP, two of five patients (40%) lacking HCV RNA in B cells achieved RVR and SVR; none of these five patients had a NVR. In contrast, none of 14 patients with positive HCV

Table 3 Stratified analysis of outcome for the IFN-based therapy (n = 100)

	IL28B SNP rs8099917	HCV RNA in B cells	Total	RVR	SVR	NVR
HCV genotype 1 (n = 77)	T/T (n = 58)	Positive	38	15/37 (41%)	23/37 (62%)	2/37 (0.5%)
		Negative	20	11/20 (55%)	14/20 (70%)	1/20 (0.5%)
	T/G, G/G (n = 19)	Positive	14	0/14 (0%)	0/14 (0%)	12/14 (86%)
		Negative	5	2/5 (40%)	2/5 (40%)	0/5 (0%)
HCV genotype 2 (n = 23)	T/T (n = 19)	Positive	4	2/4 (50%)	2/4 (50%)	0/4 (0%)
		Negative	15	12/15 (80%)	15/15 (100%)	0/15 (0%)
	T/G, G/G (n = 4)	Positive	2	0/2 (0%)	1/2 (50%)	0/2 (0%)
		Negative	2	2/2 (100%)	2/2 (100%)	0/2 (0%)

HCV, hepatitis C virus; NVR, null viral responder; RVR, rapid viral responder; SNP, single-nucleotide polymorphism; SVR, sustained viral responder