Table 4. The prevalence of the 9 drug-resistant mutations detected by ultra-deep sequencing derived from liver tissue.

	M204V/I		L180M		T184S/A/I/ L/G/C/M		S202C/G/I		I169T	
Drugs	**LAM/ETV**		**LAM/ETV**		**ETV**		**ETV**		**ETV**	
Chronic-naive										
Liver #1	**27/5421**	**(0.5%)**	2/3694	(-)	9/3886	(-)	5/5613	(-)	5/3784	(-)
Liver #2	**35/5344**	**(0.7%)**	0/538	(-)	1/563	(-)	17/6340	(-)	0/512	(-)
Liver #3	**13/1363**	**(1.0%)**	0/304	(-)	1/358	(-)	1/1379	(-)	0/264	(-)
Liver #4	11/5113	(-)	0/556	(-)	**2/547**	**(0.4%)**	11/5133	(-)	0/639	(-)
Liver #5	**2/117**	**(1.1%)**	0/409	(-)	1/380	(-)	1/189	(-)	1/474	(-)
Liver #6	12/8451	(-)	0/309	(-)	0/328	(-)	22/8457	(-)	0/334	(-)
Liver #7	**10/3098**	**(0.3%)**	1/1547	(-)	3/1477	(-)	8/3161	(-)	0/1621	(-)
Liver #8	**13/2442**	**(0.5%)**	1/2378	(-)	6/2312	(-)	1/2564	(-)	1/2507	(-)
Liver #9	**67/13879**	**(0.5%)**	2/5443	(-)	2/5107	(-)	6/13804	(-)	0/5650	(-)
Liver #10	16/7400	(-)	0/3524	(-)	3/3283	(-)	5/7113	(-)	0/3492	(-)
Liver #11	0/412	(-)	1/1328	(-)	**1/295**	**(0.3%)**	0/425	(-)	3/4729	(-)
Liver #12	**4/1098**	**(0.4%)**	1/1389	(-)	0/1272	(-)	2/1102	(-)	0/1544	(-)
Liver #13	**8/2476**	**(0.3%)**	1/2192	(-)	3/2085	(-)	4/2529	(-)	4/5029	(-)
Liver #14	5/3713	(-)	0/2009	(-)	4/1925	(-)	2/3820	(-)	5/3784	(-)
Chronic-NA										
Liver #15	0/339	(-)	0/49	(-)	0/49	(-)	0/338	(-)	0/40	(-)
Liver #16	**28/7278**	**(0.4%)**	0/4403	(-)	6/4053	(-)	14/7556	(-)	6/6084	(-)
Liver #17	**177/945**	**(18.7%)**	0/1059	(-)	0/1009	(-)	0/945	(-)	0/1051	(-)
Liver #18	**13/2655**	**(0.5%)**	0/1239	(-)	0/1185	(-)	**10/2708**	**(0.4%)**	0/1332	(-)
Liver #19	**80/6795**	**(1.2%)**	0/3168	(-)	2/2971	(-)	3/6734	(-)	0/3384	(-)

	M250V/I		A181T/V		N236T		P237H	
Drugs	**ETV**		**ADV**		**ADV**		**ADV**	
Chronic-naive								
Liver #1	**23/2719**	**(0.9%)**	10/3755	(-)	4/4210	(-)	2/4139	(-)
Liver #2	**9/2079**	**(0.4%)**	**2/549**	**(0.4%)**	1/1144	(-)	1/1188	(-)
Liver #3	**10/1699**	**(0.6%)**	**1/298**	**(0.3%)**	3/1636	(-)	1/1666	(-)
Liver #4	**3/388**	**(0.8%)**	**3/549**	**(0.5%)**	0/560	(-)	0/533	(-)
Liver #5	**2/91**	**(2.2%)**	1/409	(-)	0/55	(-)	0/60	(-)
Liver #6	0/214	(-)	**6/305**	**(2.0%)**	**1/294**	**(0.3%)**	0/257	(-)
Liver #7	**7/1289**	**(0.5%)**	4/1531	(-)	**24/2738**	**(0.9%)**	1/2692	(-)
Liver #8	2/1117	(-)	**689/2336**	**(29.5%)**	2/1713	(-)	0/1639	(-)
Liver #9	**27/7325**	**(0.4%)**	**38/5334**	**(0.7%)**	1/6607	(-)	4/6702	(-)
Liver #10	**12/3815**	**(0.3%)**	0/3454	(-)	**13/3245**	**(0.4%)**	2/3272	(-)
Liver #11	**1/199**	**(0.5%)**	1/972	(-)	0/251	(-)	0/251	(-)
Liver #12	**2/672**	**(0.3%)**	**408/1362**	**(30.0%)**	0/598	(-)	0/597	(-)
Liver #13	1/947	(-)	2/2160	(-)	0/1406	(-)	1/1374	(-)
Liver #14	**23/2719**	**(0.9%)**	10/3755	(-)	4/4210	(-)	2/4139	(-)
Chronic-NA								
Liver #15	**1/303**	**(0.3%)**	**2/49**	**(4.1%)**	0/377	(-)	0/384	(-)
Liver #16	1/922	(-)	0/4403	(-)	1/1597	(-)	3/1572	(-)
Liver #17	0/755	(-)	1/1050	(-)	0/698	(-)	**145/698**	**(20.8%)**
Liver #18	1/1464	(-)	2/1206	(-)	0/3156	(-)	0/3107	(-)
Liver #19	8/3834	(-)	**16/3128**	**(0.5%)**	0/3372	(-)	0/3428	(-)

(-): mutant clones less than 0.3% among total clones at each nucleotide sites.
LAM: lamivudine, ADV: adefovir, ETV: entecavir.
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Table 5. The prevalence of M204VI mutation at YMDD site in patients before and after entecavir administration.

	Entecavir treatment				
	Before		After		
	Prevalence of the mutated clones		Prevalence of the mutated clones		Period of NA treatment
Serum #3	222/32,238	(0.7%)	2,284/23,791	(9.6%)	2w
Serum #2	401/34,041	(1.2%)	266/25,301	(1.1%)	24w
Serum #5	521/48,723	(1.1%)	245/25,521	(1.0%)	56w
Serum #8	748/65,573	(1.1%)	336/28,702	(1.2%)	48w
Serum #9	312/30,599	(1.0%)	169/14,172	(1.2%)	56w
Serum #1	9/22,843	(-)	2,839/34,162	(8.3%)	8w
Serum #7	26/65,564	(-)	923/66,458	(1.4%)	4w
Serum #12	91/65,616	(-)	258/27,958	(0.9%)	24w
Serum #13	11/23,209	(-)	206/64,747	(0.3%)	32w
Serum #4	3/7,923	(-)	39/65,575	(-)	12w
Serum #6	52/65,582	(-)	77/55,273	(-)	16w
Serum #10	38/22,522	(-)	8/21,053	(-)	8w
Serum #11	47/43,853	(-)	5/16,520	(-)	16w
Serum #14	42/42,784	(-)	40/36,668	(-)	12w

Mutation frequency (%): the ratio of total mutant clones to total aligned coverage at each nucleotide sites.
(-): mutant clones less than 0.3% among total clones at each nucleotide sites.
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and the elucidation of other unknown mutations involved in HBe seroconversion are necessary for a better understanding of the underlying mechanisms of HBe seroconversion.

One thing to be noted is that the majority of the chronic-NA cases had extremely low levels of the G1896A pre-C mutant in their liver tissues, even though those cases were serologically positive for anti-HBe and negative for HBeAg. Moreover, entecavir administration significantly reduced the proportion of the G1896A pre-C mutant in the serum of the majority of patients irrespective of their HBeAg serostatus, while the G1896A pre-C mutant clones were detectable in a substantial proportion before treatment in all cases. These findings suggest that the G1896A pre-C mutant have higher sensitivity to NA than the wild-type viruses. Consistent with this hypothesis, several previous studies reported that NA is effective against acute or fulminant hepatitis caused by possible infection with the G1896A pre-C mutant [34,35]. Based on these findings, early administration of NA might be an effective strategy for treating patients with active hepatitis infected predominantly with the G1896A pre-C mutant.

Ultra-deep sequencing has a relatively higher sensitivity than conventional direct population sequencing and is thus useful for detecting drug-resistant mutations not detected by standard sequencing [20,21]. Recently, we revealed that drug-resistant mutants were widely present in treatment-naïve HCV-infected patients, suggesting a putative risk for the expansion of resistant clones to anti-viral therapy [19]. Here, we demonstrated that various drug-resistant HBV variants are present in a proportion of chronically HBV-infected, NA-naïve patients. Several studies using ultra-deep sequencing provided evidence that naturally-occurring drug-resistant mutations are detectable in treatment-naïve individuals with human immunodeficiency virus-1 infection [30,36,37]. Consistent with the cases of human immunodeficiency virus-1 infection, a few studies detected minor variants resistant to NA in the plasma of treatment-naïve patients with chronic HBV infection [20,21]. It remains unclear, however, whether these minor drug-resistant mutations have clinical significance. Our observation of the relative expansion of viral clones with the M204VI mutation during entecavir therapy in some cases indicates the possibility that preexisting minor mutants might provide resistance against NA through the selection of dominant mutant clones. Future studies with a larger cohort size are required to clarify the clinical implications of the latently existing low-abundant drug-resistant mutations.

The current ultra-deep parallel sequencing technology has limitations in the analyses of viral quasispecies. First, because the massively-parallel ultra-deep sequencing platform is based on a multitude of short reads, it is difficult to evaluate the association between nucleotide sites mapped to different genome regions in a single viral clone. Indeed, potential mutational linkages between the pre-C and reverse transcriptase regions were difficult to elucidate due to the short read length of the shotgun sequencing approach. Second, accurate analysis of highly polymorphic viral clones by ultra-deep sequencing is also difficult because the identification of mutations depends strongly on the mapping to the reference genome sequences.

In conclusion, we demonstrated that the majority of patients positive for anti-HBe and negative for HBeAg lacked the predominant infection of the G1896A pre-C mutant in the presence of NA treatment, suggesting that the G1896A pre-C mutant have increased sensitivity to NA therapy compared with wild-type HBV. We also revealed that drug-resistant mutants are widely present, even in the liver of treatment-naïve HBV-infected patients, suggesting that the preexisting low-abundant mutant clones might provide the opportunity to develop drug resistance against NA through the selection of dominant mutations. Further analyses utilizing both novel and conventional sequencing technologies are necessary to understand the significance and clinical relevance of the viral mutations in the pathophysiology of various clinical settings in association with HBV infection.

Supporting Information

Figure S1 Comparison of the viral complexity between the liver and serum of the same individual. Shannon entropy values throughout the whole viral genome of the liver and serum of the representative two cases are shown. (upper two panels, case #11; lower two panels, case #14). preC-C: pre-core~core, preS: pre-surface, P: polymerase.
(TIF)

Table S1 The oligonucleotide primers for amplifying HBV sequences in each clinical specimen.
(DOCX)

Table S2 Error frequency of Ultra-deep sequencing for the expression plasmid encoding wild-type genotype C HBV genome sequences by the three control experiments.
(DOCX)

Table S3 The sensitivity and accuracy of detecting the low abundant minor clones in association with the different coverage numbers.
(DOCX)

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Author Contributions

Conceived and designed the experiments: NN HM. Performed the experiments: NN HM. Analyzed the data: NN HM YU AN TF ST KS TC. Contributed reagents/materials/analysis tools: NN HM YU YO TK SY SU. Wrote the paper: NN HM YU KT TC.

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Original article

De novo activation of HBV with escape mutations from hepatitis B surface antibody after living donor liver transplantation

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Background: *De novo* activation of HBV occurs after liver transplantation from hepatitis B surface antigen (HBsAg)-negative and hepatitis B core antibody (anti-HBc)-positive donors, even under hepatitis B immunoglobulin (HBIG) prophylaxis. One reason for the activation of HBV is the emergence of HBV with escape mutations from hepatitis B surface antibody (anti-HBs). The aim of this study is to clarify the clinical features for *de novo* activation of HBV with anti-HBs escape mutations after liver transplantation.

Methods: Clinical features of 75 patients who received HBIG prophylaxis >6 months after liver transplantation with liver grafts from anti-HBc-positive donors were retrospectively analysed.

Results: Among the 75 recipients, 19 (25%) developed *de novo* activation of HBV. Of the 19 recipients, the emergence of HBV with anti-HBs escape mutations was confirmed in 7 patients. The rate of *de novo* activation of HBV with anti-HBs escape mutations was 12% at 5 years. Sequence analysis revealed mutations in the common 'a' determinant region of the surface gene, including G145R, G145A and Q129P, in HBsAg. Administration of entecavir immediately after the occurrence of *de novo* HBV activation resolved hepatitis and induced clearance of serum HBsAg and HBV DNA in all four patients receiving entecavir.

Conclusions: Escape mutations from anti-HBs caused *de novo* activation of HBV under HBIG prophylaxis after liver transplantation. Early administration of entecavir was effective on *de novo* activation of HBV with anti-HBs escape mutations.

Introduction

Most individuals who are negative for hepatitis B surface antigen (HBsAg) but positive for hepatitis B core antibody (anti-HBc) – which is indicative of resolved hepatitis B – have persistent viral infection in the liver [1,2]. We previously demonstrated that latent HBV infection is accompanied by ongoing viral replication in the liver but not in the serum or lymphatic cells of healthy anti-HBc-positive liver transplant donors [3,4]. It is possible for latently infected HBV to be transmitted from anti-HBc-positive donors to recipients via liver grafts and reactivated under the immunosuppressive conditions imposed after liver transplantation. This reactivation is called *de novo* activation of HBV [5–7].

To prevent *de novo* activation of HBV after liver transplantation, hepatitis B immunoglobulin (HBIG) had been widely used as a prophylaxis post-surgery [7–9], although lamivudine with or without HBIG has recently become the standard prophylaxis [10,11]. Even under HBIG prophylaxis, occurrence of *de novo* activation of HBV has been reported [8,12,13]. Recently, we reported that *de novo* hepatitis B occurred in 24% of HBV-naive recipients who received liver grafts from anti-HBc-positive donors [13]. Among these cases, one of the most important factors associated with HBV activation was found to be the emergence of HBV with escape mutations from hepatitis B surface antibody (anti-HBs). Escape mutations from anti-HBs occur in the common 'a' determinant region of the surface gene, which is a highly conformational region of the HBsAg protein. Mutations in and around the 'a' determinant region have been shown to alter the antigenicity of the HBsAg protein; consequently, anti-HBs fails to neutralize HBV

[14–16]. Anti-HBs escape mutations have been found in patients vaccinated for HBV [17,18], in patients with chronic hepatitis B [19,20] and in liver transplant recipients after HBIG administration [21,22]. The clinical significance of the anti-HBs escape mutant HBV has been well-analysed in patients after HBV vaccination. The prevalence of anti-HBs escape mutants after HBV vaccination was reported to have increased from 7.8% in 1984 to 19.6% in 1989; after a 1994 survey, prevalence was reported to be 28.1% [18]. Commonly reported mutations in HBsAg with the potential to escape neutralization by vaccine-induced antibody in patients after HBV vaccination include G145R, D144A, P142S, K141E, Q129H, I/T126N/A and M133L [18,23]. By contrast, the clinical features of anti-HBs escape mutants after liver transplantation under HBIG prophylaxis have not been well-analysed.

Treatment strategies for HBV with anti-HBs escape mutations have not been clarified. At present, several nucleoside analogues such as lamivudine, adefovir and entecavir are available for the treatment of chronic hepatitis B [24]. Among them, entecavir, a carbocyclic analogue of 2′-deoxyguanosine, has been shown to have higher efficacy and lower rates of resistance than lamivudine for patients with chronic hepatitis B [24]; therefore, entecavir is now used as a first-line therapy in the treatment of chronic hepatitis B worldwide. However, the efficacy of nucleoside analogues for HBV with escape mutations from anti-HBs is unknown.

The aim of this study was to clarify the clinical features of *de novo* activation of HBV with escape mutations from anti-HBs under HBIG prophylaxis after liver transplantation.

Methods

Patients

We retrospectively analysed the medical records of 157 patients who underwent living donor liver transplantation (LDLT) using liver grafts from HBsAg-negative but anti-HBc-positive donors from July 1995 to August 2008 (Figure 1A). Of these, 57 recipients were excluded from our study because their sera were pre-operatively positive for HBsAg and/or HBV DNA. An additional 25 patients were also excluded from the study because of the short duration (<6 months) of their follow-up in our hospital. Accordingly, 75 patients with a follow-up period of >6 months were enrolled in this study. The study protocol was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (Kyoto, Japan), and all patients provided informed consent.

Prophylaxis with HBIG and immunosuppressive protocol

HBIG monotherapy was given to all recipients with grafts from anti-HBc-positive donors, as reported previously [7]. The first application of HBIG at a dose of 200 IU/kg body mass was administered during the anhepatic phase of LDLT, and 100 IU/kg of HBIG was administered, if required, to maintain serum anti-HBs titres at >500 IU/l during the first post-operative month. Subsequently, HBV serological markers were examined at monthly intervals after the transplant operation, and 1,000 IU of HBIG was periodically administered to maintain serum anti-HBs titres at >200 IU/l throughout the follow-up period.

The standard immunosuppression protocol comprised tacrolimus and low-dose steroid therapy. The target whole blood lower level for tacrolimus was 10–15 ng/ml during the first 2 weeks, 10 ng/ml thereafter and 5–8 ng/ml starting from the second month. Steroid therapy was initiated at a dose of 10 mg/kg of prednisolone before graft reperfusion, then tapered down from 1 mg/kg per day on the first day to 0.3 mg/kg per day until the end of the first month, followed by 0.1 mg/kg per day until the end of the third month. After that, steroid administration was terminated.

Diagnosis of *de novo* activation of HBV

De novo activation of HBV was diagnosed when HBsAg and HBV DNA became positive in the serum of the liver transplant recipient. Serological HBV markers, including HBsAg, anti-HBs, anti-HBc, hepatitis B e antigen (HBeAg) and antibodies to HBeAg (anti-HBe), were measured by chemiluminescent enzyme immunoassay (CLEIA; Fuji Rebio, Tokyo, Japan). Serum HBV DNA titre was analysed using a commercial PCR assay (Amplicor HBV Monitor; Roche, Branchburg, NJ, USA).

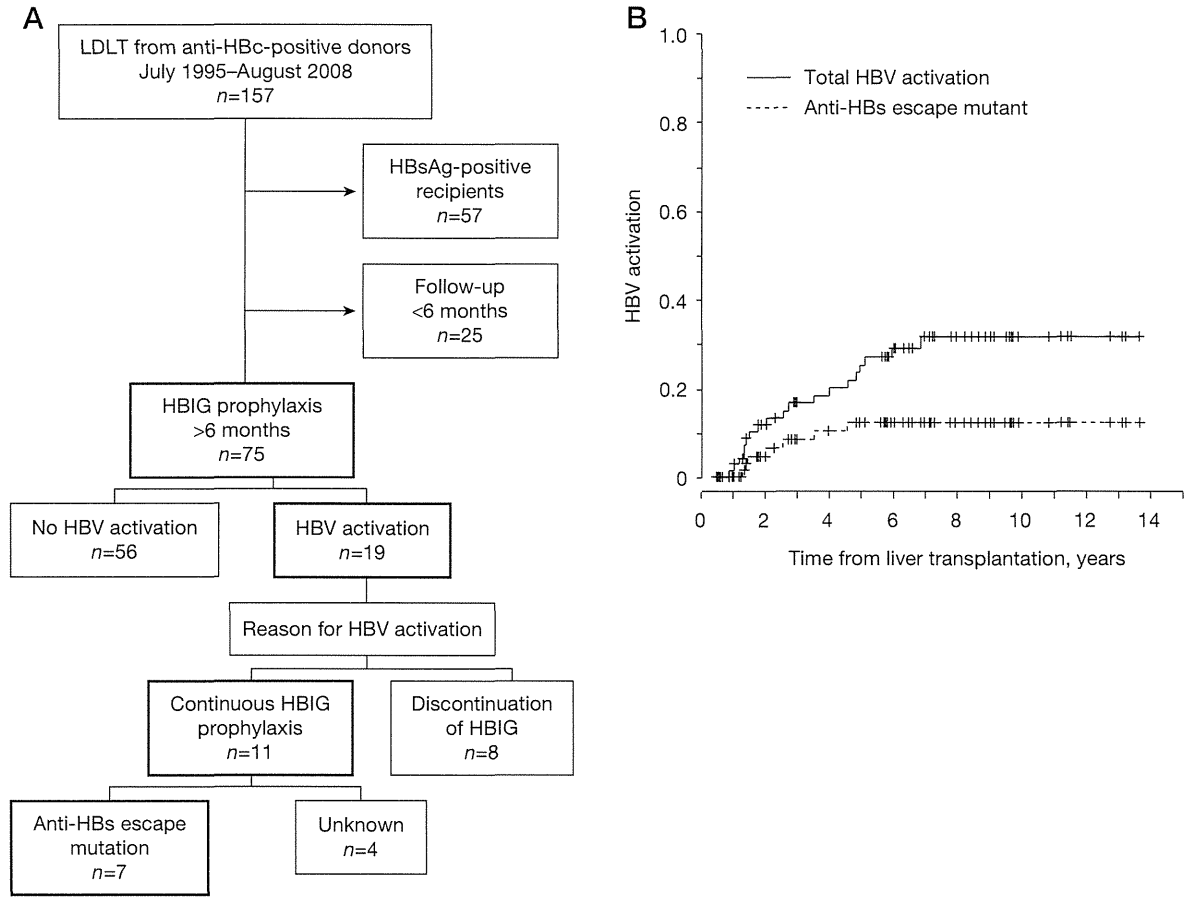
PCR amplification of HBV DNA and sequencing of the surface gene

Serum samples were obtained at the diagnosis of *de novo* activation of HBV for the analysis of HBV DNA sequencing. Preparation of DNA samples and detection of HBV genomes by PCR have been described previously [3,13]. The nucleotide sequence spanning the S region was amplified by PCR using specific primers, 5′-TGCCCTTGGATAAAGGCATT-3′ and 5′-AAGTTAAGGGAGTAGCCCCA-3′, followed by direct sequencing analyses using primers 5′-CCTGCTGGTGGCTCCAGTTC-3′ and 5′-AAGTTAAGGGAGTAGCCCCA-3′.

Statistical analysis

Baseline characteristics were tabulated and compared between patients with activation of HBV with anti-HBs escape mutations and patients without HBV activation (Table 1). For continuous variables, medians and ranges are given, and the data were analysed by the Wilcoxon rank-sum test. For categorical variables, counts

Figure 1. Flow diagram and Kaplan-Meier estimates of *de novo* activation of HBV after LDLT



(A) Flow diagram showing *de novo* activation of HBV after living donor liver transplantation (LDLT) from hepatits B core antibody (anti-HBc)-positive donors. (B) Kaplan–Meier estimates of the rate of patients who showed *de novo* activation of HBV after LDLT from anti-HBc-positive donors. The total rate and rate of activation of HBV with hepatitis B surface antibody (anti-HBs) escape mutations are shown. HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen.

are given, and the data were analysed by the χ^2 test. The rates of patients who showed HBV activation after LDLT were estimated using the Kaplan–Meier method. $P<0.05$ was considered significant.

Results

De novo activation of HBV in recipients from anti-HBc-positive donors

Among the 75 recipients who received HBIG prophylaxis >6 months after LDLT with liver grafts from anti-HBc-positive donors, 19 (25%) patients developed *de novo* activation of HBV (Figure 1A). The rate of HBV activation estimated by the Kaplan–Meier method was 3% at 1 year, 17% at 3 years, 25% at 5 years, and 29% at 10 years (Figure 1B). Of the 19 recipients with HBV activation, 8 had HBV activation due to transient discontinuation of HBIG (Figure 1A). In the remaining 11 patients with HBV activation, despite continuous HBIG prophylaxis, the emergence of HBV with anti-HBs escape mutations was confirmed in 7 patients, including 2 patients who were described in our previous report [13]. The rate of *de novo* activation of HBV with anti-HBs escape mutations estimated by the Kaplan–Meier method was 8% at 3 years and 12% at 5 years (Figure 1B). The other four recipients, in whom the reason for HBV activation was unknown, were previously reported by us [13].

Clinical features of patients with *de novo* activation of HBV with anti-HBs escape mutations

To clarify the characteristics of patients with *de novo* activation of HBV with anti-HBs-escape mutations, the clinical features of recipients with HBV with anti-HBs escape mutations ($n=7$) were listed and compared

Table 1. Clinical features of patients with *de novo* activation of HBV with anti-HBs escape mutation, of those without HBV activation after liver transplantation and also of donors

Characteristic	Activation of HBV with anti-HBs escape mutations (n=7)	No HBV activation (n=56)	P-value
Recipient			
Age, years	17 (0–67)	12 (0–62)	0.433[a]
Male/female	3/4	21/35	1.000[b]
Primary disease			
Cholestatic diseases	4 (57)	31 (55)	–
Hepatocellular diseases	0	8 (14)	–
Neoplastic diseases	1 (14)	6 (11)	–
Acute liver failure	0	1 (2)	–
Metabolic diseases	0	3 (5)	–
Retransplantation	1 (14)	6 (11)	–
Other	1 (14)	1 (2)	–
HBV markers before LDLT			
Anti-HBs-positive	3 (43)	18 (32)	0.677[b]
Anti-HBe-positive	0	7 (13)	0.581[b]
Anti-HBc-positive	0	17 (30)	0.175[b]
Donor			
Age, years	42 (34–55)	45 (24–65)	0.200[a]
Male/female	3/4	31/25	0.694[b]
Anti-HBs-positive	7 (100)	44 (79)	0.329[b]
Anti-HBe-positive	4 (57)	38 (68)	0.419[b]
Follow-up period, months	86 (48–151)	94 (12–180)	0.646[a]

Qualitative variables are displayed as n (%) and quantitative variables expressed as median (range) for non-normally distributed variables. [a]Wilcoxon rank-sum test. [b]χ^2 test. Anti-HBc, heptatitis B core antibody; anti-HBe, hepatitis B e antibody; anti-HBs, hepatitis B surface antibody; LDLT, living donor liver transplantation.

Table 2. Clinical features of seven patients with *de novo* activation of HBV with anti-HBs escape mutation after liver transplantation

Patient number	Age, years[a]	Sex	Months from LDLT to HBV activation	Anti-HBs titre before HBV activation, IU/l	At the time of HBV activation			
					Anti-HBs titre, IU/l	HBsAg, COI	Serum HBV DNA, copies/ml	Peak ALT, IU/l
1	12	F	16.8	418.4	275.2	>2,000	$>10^{7.6}$	410
2	22	M	54.9	27.8	16.6	60.4	$10^{6.6}$	1,300
3	26	F	17.9	284.5	82.6	10.2	$>10^{7.6}$	81
4	70	F	42.2	44.0	17.9	668.8	$10^{7.1}$	204
5	59	M	24.1	188.8	105.4	21.3	$10^{3.5}$	131
6	18	F	30.6	96.9	157.4	6.9	$10^{4.5}$	153
7	2	M	15.7	218.8	74.3	187.3	NE	111

[a]Age at HBV activation. ALT, alanine aminotransferase; anti-HBs, hepatitis B surface antibody; COI, cutoff index; F, female; HBsAg, hepatitis B surface antigen; LDLT, living donor liver transplantation; M, male; NE, not examined.

with those of recipients without HBV activation (n=56; Table 1). The two groups of patients did not differ significantly by age, sex or serological markers for HBV before LDLT with regard to either recipients or donors. Of note, all seven patients with *de novo* activation of HBV with anti-HBs escape mutations were negative for anti-HBc pre-operatively, and no anti-HBc-positive recipients (n=17) developed *de novo* activation of HBV.

The details of the clinical features of the seven patients who developed *de novo* activation of HBV with anti-HBs-escape mutations are summarized in Table 2. In the seven patients, serum HBsAg and HBV DNA became positive 15.7–54.9 months (median 24.1 months) after LDLT. Serum anti-HBs titres were maintained at 27.8–418.4 IU/l before HBV activation by HBIG administration. At the time of HBV activation, all patients were positive for anti-HBs, despite being positive for serum HBV DNA and HBsAg, when *de novo* hepatitis B was diagnosed. The genotype of HBV in all seven patients was C, which is the major genotype in Japan [25]. All patients showed high serum ALT levels. Five of the seven patients received tacrolimus only for immunosuppression at the time of HBV activation, patient number 3 had tacrolimus with

prednisolone and mycophenolate mofetil, and patient number 4 received cyclosporine and prednisolone.

Sequence analysis of serum HBV DNA

Results of the sequence analysis of serum HBV DNA in these seven patients are shown in Figure 2. We focused our analysis on the immunodominant loop encompassing amino acid (aa) 101–163 of the S protein, which includes the 'a' determinant region (aa 124–147), the major target of neutralizing anti-HBs antibodies due to its exposure at the surface of viral particles [14,16,26]. Sequencing of the S gene revealed the presence of mutations in the immunodominant loop in all patients. These mutations within the S protein led to G145A substitution in patient number 1, G145R substitution in patients numbered 2, 3, 4, 6 and 7, and Q129P substitution in patient number 5 (Figure 2B). The mutations at aa 145 or aa 129 in HBsAg are known to be escape mutations from anti-HBs [9,16,18,22]. Several other mutations with amino acid substitutions, whose roles have not yet been clarified, were found at aa 101, aa 103, aa 110, aa 113, aa 114, aa 120, aa 126, aa 143, aa 155 and aa 161.

Treatment for *de novo* activation of HBV with anti-HBs escape mutations

Entecavir treatment (0.5 mg) was started for four patients (numbers 1–4) immediately after the diagnosis of *de novo* activation of HBV with anti-HBs-escape mutations. After the administration of entecavir, serum HBsAg and HBV DNA promptly decreased and became undetectable at 2.5, 3.3, 6 and 2.5 months after the start of entecavir treatment, respectively (Figure 3 and Table 3). Serum ALT levels also decreased in association with the decrease in serum HBV DNA. After confirming the stable negativity of HBsAg, entecavir treatment was stopped in three patients (numbers 1–3) at 5.8, 5.9 and 9.9 months after beginning the treatment, respectively. Thereafter, serum HBsAg and HBV DNA remained negative during the follow-up periods of 22.2, 24.7 and 20.6 months after withdrawal of entecavir, respectively (Table 3). Entecavir administration was continued for patient number 4 at the time of the analysis for this study because the patient wanted to continue the treatment. Patients numbered 5 and 6 received early administration of lamivudine after the diagnosis of *de novo* activation of HBV. However, they did not achieve serum HBV clearance by 26.6 and 4.6 months, respectively, at which time adefovir was added. Even after treatment with a combination of lamivudine and adefovir for 33.2 months, patient number 5 remained chronically HBsAg-positive. In patient number 6, serum HBsAg and HBV DNA became negative at 9.5 months after adefovir administration. Patient number 7 who did not receive nucleoside analogue treatment for hepatitis B developed chronic hepatitis B as confirmed by liver histology.

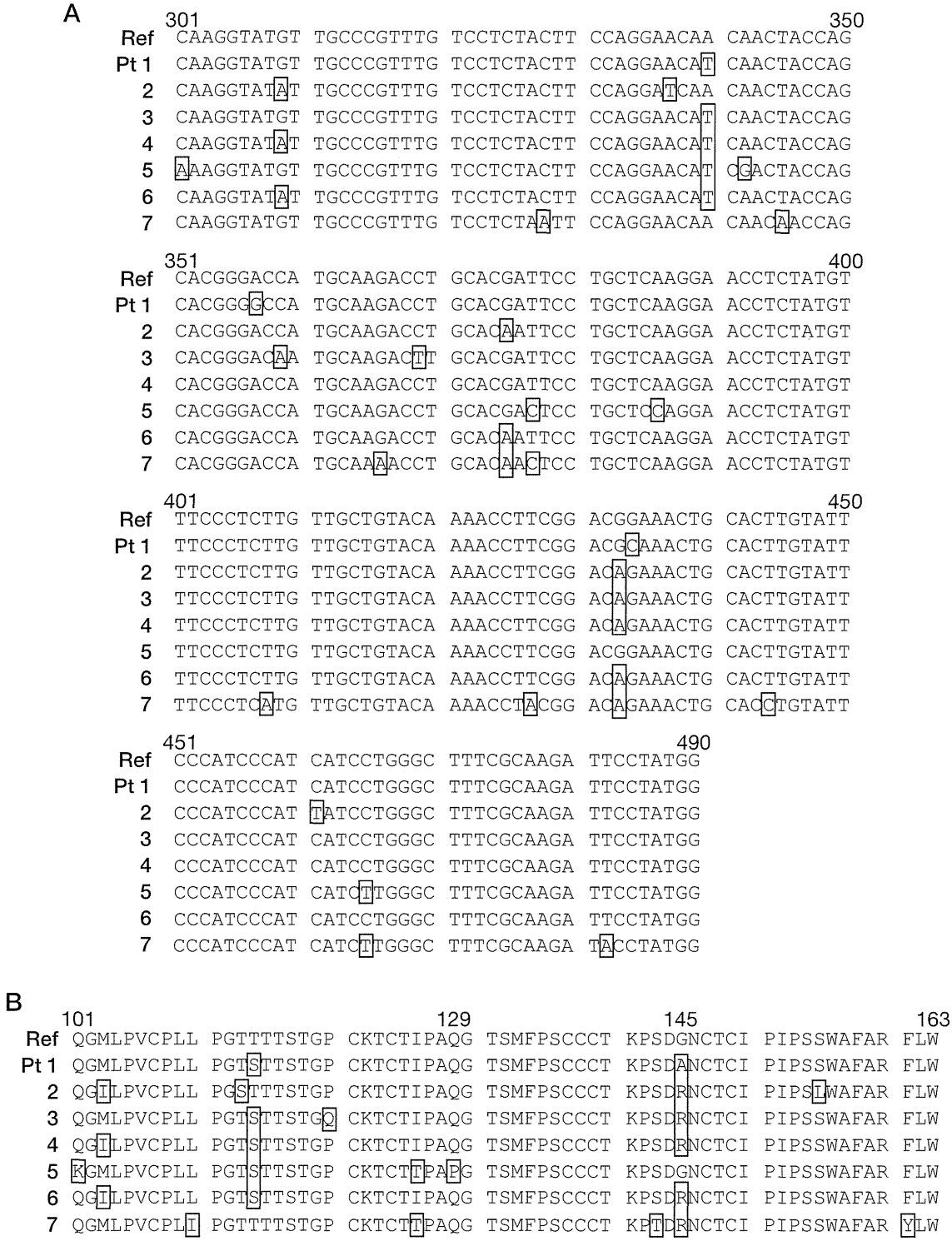
Discussion

In this report, we demonstrated the clinical features of *de novo* activation of HBV with anti-HBs-escape mutations under HBIG prophylaxis after liver transplantation. The rate of *de novo* activation of HBV with anti-HBs escape mutations was 12% at 5 years. No significant difference of baseline characteristics between patients with *de novo* activation of HBV with anti-HBs-escape mutations and patients without HBV activation was identified, but all patients who had activation of anti-HBs escape mutant HBV were pre-operatively anti-HBc-negative. Early entecavir treatment was very effective for patients with *de novo* activation of HBV with anti-HBs escape mutations and the treatment induced complete clearance of serum HBsAg and HBV DNA and resulted in sustained negativity for HBsAg even after the termination of entecavir treatment.

Two reasons for *de novo* activation of HBV after LDLT from anti-HBc-positive donors were revealed in this study: discontinuation of HBIG and emergence of an anti-HBs escape mutant. However, the reason for *de novo* HBV activation in four patients is still unknown. HBV activation by HBIG discontinuation is preventable by careful follow-up to reduce non-compliance of HBIG use. The most important reason for HBV activation is emergence of anti-HBs escape mutations under HBIG prophylaxis, because it is unpredictable and difficult to prevent. In the present study, no anti-HBc-positive patients developed *de novo* activation of HBV with anti-HBs-escape mutations. The reason for this is also unknown, but we expect that individuals with resolved hepatitis B have memory T-cells and various antibodies for HBV, including antibodies against PreS1 and PreS2 as well as anti-HBs, and the T-cells and antibodies could inhibit the proliferation of HBV with anti-HBs-escape mutations.

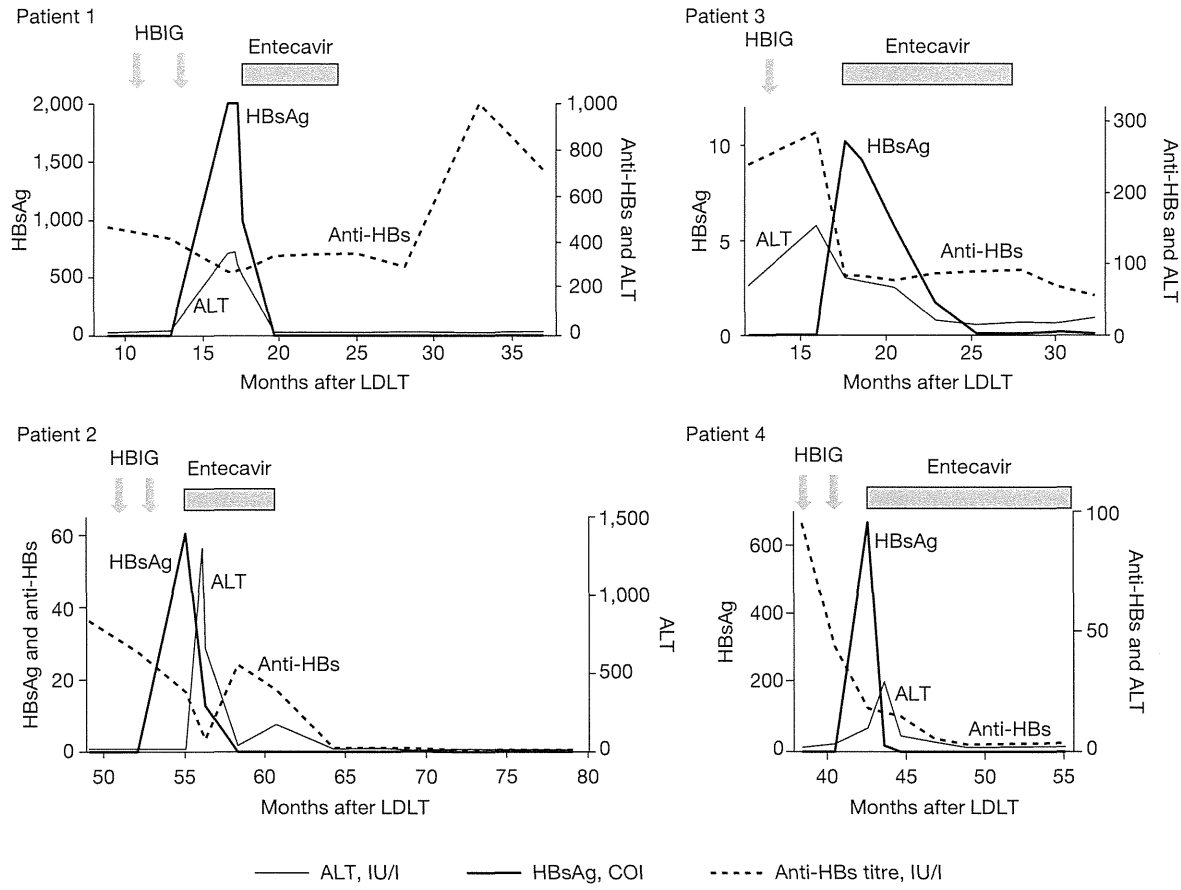
A characteristic serological feature at the onset of *de novo* activation of HBV with anti-HBs-escape mutations was positivity of anti-HBs at the time of HBV DNA appearance in the serum. Because anti-HBs cannot bind HBsAg that has anti-HBs escape mutations [16], HBV will increase even in the presence of anti-HBs. Therefore, we must be cautious to the development of hepatitis B due to HBV with anti-HBs-escape mutations, even when serum anti-HBs titre is maintained at a high level by HBIG administration. A regular evaluation of serum HBsAg and/or HBV DNA is recommended. Duration from liver transplantation to activation of anti-HBs-escape mutants were 15.7–54.9 months in this study. The previous study reported that activation of HBV with

Figure 2. DNA and amino acid sequences from seven patients with anti-HBs escape mutations



(A) DNA sequences between nucleotide 301 and 490 of the *S* gene and (B) amino acid sequences between positions 101 and 163 of the S protein of HBV from seven patients (Pt) with hepatitis B surface antibody (anti-HBs) escape mutations. The sequences are aligned with HBV genotype C (subtype adr) reference sequence (Ref; AB033550 [32]). Boxes indicate the positions showing differences from the Ref.

Figure 3. Clinical course of four patients who received entecavir treatment for *de novo* activation of HBV with anti-HBs escape mutations



The administration of hepatitis B immunoglobulin (HBIG) is shown as arrows, and treatments with entecavir are indicated as shaded boxes. ALT, alanine aminotransferase; anti-HBS, hepatitis B surface antibody; COI, cutoff index; HBsAg, hepatitis B surface antigen; LDLT, living donor liver transplantation.

anti-HBs-escape mutations in HBsAg-positive recipients occurred 1–20 months after liver transplantation [22]. According to these results, the regular evaluation of HBV should be initiated just after liver transplantation and continued for the patient's lifetime.

The natural clinical course after *de novo* activation of HBV without nucleoside analogue treatment has been revealed in previous reports. We reported on a total of 19 cases of *de novo* activation of HBV after LDLT [7,13] without treatment after HBV activation. Overall, 16 of the 19 (84%) recipients, including 1 patient with anti-HBs escape mutant HBV, became HBsAg carriers; 1 died of fibrosing cholestatic hepatitis and only 2 patients spontaneously resolved to an HBsAg-negative state. Similar results showing that a majority of patients suffering from *de novo* activation of HBV after liver transplantation developed liver cirrhosis or chronic hepatitis have been reported [5,6,27]. These results indicate that most patients with *de novo* activation of HBV after liver transplantation enter an HBsAg carrier state without anti-HBV treatment because of the immunosuppressive conditions. Therefore, an effective management strategy is required for patients with *de novo* activation of HBV after liver transplantation.

We recently reported the beneficial effects of short-term lamivudine treatment for *de novo* activation of HBV caused by reasons other than anti-HBs escape mutations [13]. Lamivudine administration during the acute phase of *de novo* activation of HBV resulted in complete clearance of HBsAg from the serum in five of six patients, and all five remained negative for HBsAg even after the termination of lamivudine treatment. However, as shown in the present study, clearance of

Table 3. Treatment for seven patients with *de novo* activation of HBV with anti-HBs escape mutation after liver transplantation

Patient number	Treatment	Present status	Follow-up period after HBV activation, months	Duration from initiation of NA to disappearance of HBsAg, months	Duration of NA treatment, months	Present treatment
1	Entecavir	Resolved	28.0	2.5	5.8	None
2	Entecavir	Resolved	30.6	3.3	5.9	None
3	Entecavir	Resolved	30.5	6	9.9	None
4	Entecavir	Resolved	18.9	2.5	18.9	Entecavir
5	Lamivudine plus adefovir	Chronic hepatitis	59.8	-	59.8	Lamivudine plus adefovir
6	Lamivudine plus adefovir	Resolved	67.8	14.1	67.8	Adefovir
7	None	Chronic hepatitis	125.1	-	-	None

Anti-HBs, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; NA, nucleoside analogue.

HBsAg was not achieved in two patients with HBV with anti-HBs escape mutation by lamivudine administration. Although one patient achieved clearance of HBsAg after administration of adefovir, another patient developed chronic hepatitis B despite adding adefovir. In contrast, we demonstrated here the potent efficacy of entecavir on HBV with anti-HBs-escape mutations. The reason for the difference in efficacy between lamivudine and entecavir is unclear. Recent reports indicate that the overlap of the gene encoding HBsAg by the polymerase gene creates a unique situation in which a change within the polymerase gene following nucleoside analogue treatment might result in structural changes in the HBsAg protein and a subsequent reduction in the antigenicity of the protein. Lamivudine-resistant mutations in the polymerase gene are, indeed, associated with changes in the HBsAg protein, with a consequent reduction in antigenicity of the HBsAg protein comparable to that of anti-HBs-escape mutants [28,29]. The reverse might also be true. It has been reported that anti-HBs-escape mutations can produce a functionally significant alteration in the viral polymerase and influence the viral replication phenotype [30]. Both entecavir and lamivudine are nucleoside analogues that inhibit the HBV polymerase, but the mechanism of inhibition is different between these two nucleoside analogues. Entecavir inhibits HBV replication at three different steps: the priming of HBV DNA polymerase, reverse transcription of the negative-strand HBV DNA from the pregenomic RNA, and synthesis of the positive-strand HBV DNA, whereas lamivudine lacks the effect of the priming of HBV DNA polymerase [31]. The difference in the mechanism of HBV polymerase inhibition between entecavir and lamivudine may contribute to the difference in the efficacy of each nucleoside analogue on HBV with anti-HBs-escape mutations.

In conclusion, escape mutations from anti-HBs caused *de novo* activation of HBV under HBIG prophylaxis after liver transplantation from donors with resolved hepatitis B. Early administration of entecavir is important to avoid the subsequent development of acute liver failure or chronic hepatitis caused by *de novo* activation of HBV with anti-HBs-escape mutants.

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Disclosure statement

The authors declare no competing interests.

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Genetic Heterogeneity of Hepatitis C Virus in Association with Antiviral Therapy Determined by Ultra-Deep Sequencing

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Abstract

Background and Aims: The hepatitis C virus (HCV) invariably shows wide heterogeneity in infected patients, referred to as a quasispecies population. Massive amounts of genetic information due to the abundance of HCV variants could be an obstacle to evaluate the viral genetic heterogeneity in detail.

Methods: Using a newly developed massive-parallel ultra-deep sequencing technique, we investigated the viral genetic heterogeneity in 27 chronic hepatitis C patients receiving peg-interferon (IFN) α2b plus ribavirin therapy.

Results: Ultra-deep sequencing determined a total of more than 10 million nucleotides of the HCV genome, corresponding to a mean of more than 1000 clones in each specimen, and unveiled extremely high genetic heterogeneity in the genotype 1b HCV population. There was no significant difference in the level of viral complexity between immediate virologic responders and non-responders at baseline ($p = 0.39$). Immediate virologic responders ($n = 8$) showed a significant reduction in the genetic complexity spanning all the viral genetic regions at the early phase of IFN administration ($p = 0.037$). In contrast, non-virologic responders ($n = 8$) showed no significant changes in the level of viral quasispecies ($p = 0.12$), indicating that very few viral clones are sensitive to IFN treatment. We also demonstrated that clones resistant to direct-acting antivirals for HCV, such as viral protease and polymerase inhibitors, preexist with various abundances in all 27 treatment-naïve patients, suggesting the risk of the development of drug resistance against these agents.

Conclusion: Use of the ultra-deep sequencing technology revealed massive genetic heterogeneity of HCV, which has important implications regarding the treatment response and outcome of antiviral therapy.

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Introduction

Hepatitis C virus (HCV) is classified as a member of the Flaviviridae family [1] and has an approximately 9.6-kb single-stranded RNA genome. This RNA genome encodes a large precursor polyprotein, which is cleaved by viral and host proteases to generate at least 10 functional viral proteins; core, envelope (E)-1, E2, p7, nonstructural protein (NS)-2, NS3, NS4A, NS4B, NS5A, and NS5B [2,3]. A strong characteristic of HCV infection is its significant genetic diversity, the consequence of the absence of proofreading activity in RNA-dependent RNA polymerase [4], and the high level of viral replication during its life cycle [5]. The mean frequency of nucleotide alterations occurring in HCV RNA is calculated to be between 1.4×10^{3} and 1.9×10^{3} substitutions per nucleotide per year [6,7]. As a result, the infecting HCV clones in each patient invariably show population diversity with a high degree of genetic heterogeneity. The collection of viruses in a population of closely related but non-identical genomes is referred to as a quasispecies [8,9], and the dominant viral population may be evolving as a result of its viral replicative fitness and concurrent immune selection pressures that drive clonal selection.

It is reasonable to assume that the viral pathogenesis and sensitivity to treatment are affected by the generation of escape mutants through immune evasion and the modification of virulence characteristics by anti-viral treatment [10]. Thus, certain viral mutations have important implications for the pathogenesis of the viral disease and the sensitivity to antiviral therapy. Several studies have attempted to associate genetic heterogeneity or

number of mutations with pathogenesis and treatment outcome. However, the abundant diversity and complexity of the chronically-infected HCV has been an obstacle to evaluate the viral genetic heterogeneity in detail. In this respect, the recent introduction of ultra-deep sequencing technology, capable of producing millions of DNA sequence reads in a single run, is rapidly changing the landscape of genome research [11,12]. One application of ultra-deep sequencing was the identification of rare minority drug resistant clones of human immunodeficiency virus, which are not detectable by standard sequencing techniques [13–15]. Moreover, the recent study using 454/Roche pyrosequencing technology clarified the transmission bottlenecks by measuring the population structure within patients with HCV infection [16].

In this study, we used for the first time ultra-deep sequencing with Illumina Genome Analyzer II (Illumina, San Diego, CA) and determined the pictures of viral quasispecies of genotype 1b HCV in patients receiving peg-interferon (IFN) α2b plus ribavirin (RBV) to clarify the significance of the viral genetic complexity in the pathophysiology of HCV infection and the treatment outcome of the current IFN-based therapy for HCV-infected patients. Because our main objective was to determine whether the HCV sequence variation itself is responsible for the sensitivity or resistance to antiviral therapy, we compared the composition of the HCV population complexity 1 week after IFN administration in patients who showed a prompt decrease in HCV viremia with those in whom there was no reduction in the serum HCV RNA levels after the initiation of IFN treatment. We also examined the prevalence of drug-resistant mutations to direct-acting antivirals (DAAs) for HCV in treatment-naïve HCV-infected patients, based on the fact that drug-resistant mutations already exist in treatment-naïve patients with various pathogenic virus infections, such as human immunodeficiency viruses [14,17].

Results

Validation of multiplex ultra-deep sequencing of the HCV genome

We performed a massive parallel ultra-deep sequencing run on the Illumina Genome Analyzer II platform using multiplex tagging methods. First, we conducted a control experiment to validate the efficacy and error rates in ultra-deep sequencing of the viral genome. For this purpose, we used a plasmid encoding full-length HCV [18] as a template and determined the plasmid-derived whole HCV sequence. The ultra-deep sequencing platform provided us the full-length HCV genome information derived from the plasmids with a mean coverage of 1674.3 at each nucleotide site (Table 1). Errors comprised insertions (1.0%), deletions (4.2%), and nucleotide mismatches (94.8%) and the overall error rates by multiplex ultra-deep sequencing were determined to be a mean of 0.0010 per bp. Next we confirmed that the high-fidelity PCR amplification with HCV-specific primer sets followed by multiplex ultra-deep sequencing resulted in no significant increase in the error rates in the viral sequencing data (ranging from 0.0012 to 0.0013 per bp; per-nucleotide error rate, 0.12%–0.13%).

To estimate the accuracy of detecting nucleotide alterations using reads filtered by average base quality and mapping quality, we introduced the plasmid with single point mutations within the wild-type viral sequences with the ratio of 1:99 and 1:999 and assessed the sensitivity and accuracy of quantification with the high-fidelity PCR amplification followed by multiplex ultra-deep sequencing. Duplicate control experiments revealed that mutations present at an input ratio of 0.10% ranged between 0.09 and 0.19%, and the results could be reproducibly quantified (data not shown). Based on these results, we picked up the low abundant mutations that presented at frequency of more than 0.20% among the total viral clones, a level that could rule out putative errors caused by massively-parallel sequencing, in the current platform used in this study.

Table 1. Error frequency of ultra-deep sequencing for the plasmid encoding full-genome HCV sequence.

	PCR amplification	
	(−)*	(+)*
Total read nucleotides	15,118,929	24,158,372
Mean coverage	1674.3	5562.6
Type of errors		
mismatches	14,629 (94.8%)	26,243 (88.6%)
deletions	640 (4.2%)	2510 (8.5%)
insertions	147 (1.0%)	859 (2.9%)
Overall error rate (%)	0.102	0.123

*(−); Ultra-deep sequencing of HCV encoding plasmid
(+); Ultra-deep sequencing of PCR-amplified HCV encoding plasmid.
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Large heterogeneity of viral clones in HCV-infected patients

HCV infection comprises a heterogeneous mixture of viral clones with various mutations. To clarify the landscape of HCV heterogeneity as a quasispecies, we determined the viral full-genome sequences derived from 27 HCV-infected patients by multiplex ultra-deep sequencing and compared the results with those obtained by the direct population Sanger sequencing method. All sequence reads by multiplex ultra-deep sequencing have been deposit in DNA Data Bank of Japan Sequence Read Archive (http://www.ddbj.nig.ac.jp/index-e.html) under accession number DRA000366.

HCV nucleotide sequence reads by ultra-deep sequencing were aligned to the consensus viral sequences in the same serum specimen that were determined by direct population Sanger sequencing. A mean number of 1705-fold coverage on average was achieved at each nucleotide site of the HCV sequences in each specimen. The average frequencies of altered sequences detected in each viral genomic region are summarized in Table 2. Compared with the representative sequence of the population average clone, the mutation frequency was 1.04% of the total viral genomic sequences and 16.1% of the total nucleotide positions on average. Most of the genomic changes observed in viral variants were single base substitutions and unevenly distributed throughout the region of the HCV genome.

Among the viral genomic regions, the nucleotide sequence complexity expressed as the Shannon entropy was smallest in the core region. In contrast, the viral sequence complexity in the E2 region was highest among the HCV genomic regions and significantly greater than the average mutation frequency of the remaining HCV genome ($p = 0.0026$). Similarly, the ratio of the number of mutated nucleotides to the total number of nucleotides analyzed in the E2 region was significantly higher than that of the remaining HCV genome ($p = 5.66 \times 10^{-6}$). These findings clearly confirmed that the quasispecies complexity in E2, which contains hypervariable region1 (HVR1) and HVR2, was prominently larger than that of other viral genomic regions [19].

Table 2. Mean genetic complexity of the genotype1b HCV in chronically infected 27 patients.

Viral genomic Region	Mean number of aligned nucleotides	Mean number of mutated nucleotides	Mean coverage	Mutation frequency (%)	Mean Shannon entropy
Core	779,839	5027	1361	0.61	0.045926
E1	739,220	7902	1360	0.99	0.064884
E2	1,382,907	19,724	1265	1.37	0.088584
p7	217,000	3237	1148	1.44	0.075829
NS2	673,579	8702	1073	1.19	0.075333
NS3	4,958,188	52,204	2619	0.93	0.060767
NS4A	427,677	5604	2640	1.32	0.072217
NS4B	1,209,000	17,485	1544	1.26	0.063190
NS5A	2,034,626	28,820	1518	1.28	0.067398
NS5B	2,720,417	27,449	1681	0.90	0.054805
Total	14,875,801	172,327	1705	1.04	0.062624

doi:10.1371/journal.pone.0024907.t002

Early dynamic changes of viral complexity after the administration of peg-IFNα2b plus RBV

Among 27 patients enrolled in this study, 8 showed a prompt decrease in their serum HCV RNA levels and 8 showed no significant changes 1 week after initiating treatment with peg-IFNα2b plus RBV. To clarify the changes in the viral quasispecies in response to antiviral therapy, we determined the early dynamic changes in viral complexity before and after 1 week of peg-IFNα2b plus RBV administration in these 8 immediate virologic responders and 8 non-responders. All cases were infected with genotype 1b viruses, and the clinical features, including serum HCV RNA level at baseline, did not significantly differ between immediate virologic responders and non-responders (Table 3). A mean coverage of 1798-fold and 2416-fold were mapped to each reference sequence in immediate virologic responders before and after peg-IFNα2b plus RBV administration, respectively. Similarly, a mean coverage of 1780-fold and 2461-fold were determined in non-responders before and after peg-IFNα2b plus RBV administration, respectively (Table 4 and Table S1).

We then estimated the genomic complexity by calculating the Shannon entropy for each nucleotide position before and after the administration of peg-IFNα2b plus RBV (Table 4). There was no significant difference in the level of viral complexity between immediate virologic responders and non-responders at a baseline (mean Shannon entropy value 0.072 vs 0.075, $p = 0.39$). Immediate virologic responders, however, showed a significant reduction in the nucleotide sequence complexity after the administration of peg-IFNα2b plus RBV (mean Shannon entropy value 0.072 vs 0.049, $p = 0.037$), indicating that the viral quasispecies nature after the peg-IFNα2b plus RBV treatment

Table 3. Characteristics of patients that showed immediate virologic response or non-response to PEG-IFNα2b plus ribavirin combination therapy.

	Immediate virologic responders	Non-responders	P-value
Age†	50.5 (45–68)	60 (55–69)	0.12
Sex (male/female)	5/3	5/3	1
Alanine aminotransaminase† (IU/l)	54 (15–198)	72 (30–143)	0.51
Total bilirubin† (mg/dl)	0.6 (0.4–1.8)	0.8 (0.4–1.4)	0.34
Platelet count† ($\times 10^4/mm^3$)	18.9 (7.1–27.2)	16.7 (11.6–22.5)	0.68
HCV genotype	1b	1b	
HCV viral load† (log IU/ml)			
pre-treatment	6.6 (6.2–7.5)	6.9 (6.1–7.6)	0.43
after treatment	4.6 (4.0–5.2)	6.5 (6.1–6.8)	**0.028**
Final outcome			**0.025**
sustained viral response	6	0	
Relapse	1	1	
non-response	0	6	
withdraw*	1	1	

† Values are median (range).

* The treatment was discontinued in one immediate virologic responder and one non-responder, due to the side effect of IFN and the development of liver cancer, respectively.

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Table 4. Genetic complexity at pre-treatment and 1 week after PEG-IFNα2b plus ribavirin combination therapy in immediate virologic responders and non-responders.

	Immediate virologic responders (N = 8)		Non-responders (N = 8)	
	Pre-treatment	1 week after IFN therapy	Pre-treatment	1 week after IFN therapy
Mean number of aligned reads	263,452	356,963	256615	354,398
Mean number of aligned nucleotides	16,632,186	22,438,125	16,248,820	22,379,922
Mean coverage	1798	2416	1780	2461
Mutation frequency (%)	0.96	0.63	1.13	1.11
Shannon entropy	0.072*	0.049*	0.075**	0.066**

Wilcoxon rank sum test.
* $p = 0.037$.
** $p = 0.12$.
doi:10.1371/journal.pone.0024907.t004

became relatively more homogeneous than at baseline status in this group. In contrast, no significant changes in the nucleotide sequence complexity were observed in non-responder patients before and after treatment with peg-IFNα2b plus RBV (mean Shannon entropy value 0.075 vs 0.066, $p = 0.12$). We then examined whether specific nucleotide position might be associated with the response to peg-IFNα2b plus RBV treatment in immediate virologic responders, but complexity was not commonly shared at any specific nucleotide position that changed by more than 50% after peg-IFNα2b plus RBV administration (data not shown), indicating no association between the specific nucleotide position and the response to peg-IFNα2b plus RBV treatment.

Elimination of minor viral clones by peg-IFNα2b plus RBV therapy

Next, we compared the nucleotide complexity in each viral genomic region of the immediate virologic responders with that of non-responders before and after peg-IFNα2b plus RBV administration (Figure 1 and Table S2). In immediate virologic responders, the peg-IFNα2b plus RBV therapy induced a significant reduction in the nucleotide sequence complexity in all viral genomic regions except NS4B. In contrast, non-responders showed no significant change in the viral sequence complexity in any viral genomic region. For example, there was no significant difference in the mean complexity in the E2 region at baseline between the immediate virologic responders and non-responders. The administration of peg-IFNα2b plus RBV significantly reduced the levels of nucleotide sequence complexity in the E2 region in all the immediate virologic responders (mean Shannon entropy value 0.139 vs 0.085, respectively. $p = 0.012$, Figure 1 and Table S2). In contrast, no significant changes in the sequence complexity were observed in the E2 (mean Shannon entropy value 0.083 vs 0.082, respectively. $p = 0.89$) regions in non-responder cases after treatment with peg-IFNα2b plus RBV.

To examine whether certain viral clones in non-responders showed sensitivity to IFN therapy, we investigated the sequence complexity in HVR1 in the E2 region in detail before and after peg-IFNα2b plus RBV therapy, because the HVR1 region possessed one of the highest complexities among viral genomic regions. In immediate virologic responders, the heterogeneity at each nucleotide position was reduced in response to peg-IFNα2b plus RBV administration (representative nucleotide changes are shown in Figure 2A). In contrast, the ratio of mutated clones among the total sequence reads determined at each nucleotide site in HVR1 showed no significant change before and after the administration of peg-IFNα2b plus RBV in the majority of non-responders (Figure 2B), suggesting that very few viral clones showed sensitivity to peg-IFNα2b plus RBV and were eliminated after the administration of peg-IFNα2b plus RBV.

Detection of viral clones with drug-resistant mutations

Because none of the DAAs for HCV were approved by Japanese health coverage at the time of this study, all patients enrolled into this study were naïve to DAAs for HCV including protease and polymerase inhibitors. Thus, we determined whether the reported drug-resistant mutants exist spontaneously in nature among treatment-naïve HCV-infected patients. For this purpose, we examined the naturally prevalent mutations against HCV protease and polymerase inhibitors in the 27 patients. The drug-resistant mutations examined here included 9 mutations resistant to NS3/4 protease inhibitors, including Telaprevir, Boceprevir, TMC435350, ITMN191/R7227, MK-7009, and BI-201335, and 5 mutations resistant to NS5B polymerase inhibitors, including Filibuvir, BI-207127, and R7128 [20].

The mean number of sequence reads at the nucleotide position comprising mutations resistant to NS3/4A protease and NS5B polymerase inhibitors among the 27 cases were obtained with 1179-fold and 1972-fold coverage, respectively. Based on the detection rate of the low-level viral clones determined by the control experiments, we picked up the drug-resistant mutants that presented at a frequency of more than 0.2% among the total viral clones. Based on these criteria, at least one resistant mutation was detected in all subjects (Table 5). The mean prevalence of the 14 drug-resistant mutations ranged from 0.20% to 99.1% indicating that the proportion of resistant mutations substantially differed in each case. The T54S/A mutation resistant to Teraprevir and Boceprevir in genotype 1b HCV [21] was the most commonly detected (20 of 27 cases, 74.1%). The proportion of T54S/A mutations among the total clones ranged from 0.21% to 86.9% and thus substantially differed between cases. Other mutations resistant to the NS3/4A protease-inhibitor were detected in 16 of 27 cases (59.3%) at V55A and Q80R/K, and 12 of 27 cases (44.4%) at V36A/M. In contrast, no D168A/V/T/H mutation resistant to ITMN191/R7227, MK-7009, TMC435350, and BI-201335 was detectable. Regarding NS5B polymerase inhibitors, the V499A mutation resistant to BI-207127, was most frequently detected and 20 of 27 (74.1%) of subjects possessed the resistant-mutant clones at levels 0.20% to 99.1% at baseline. Only one case had the BI-207127-resistant P496A mutant clones and none had the R7128-resistant S282T clones. Of the 27 subjects, 16 (59.3%) harbored mutations resistant to at least four kinds of NS5B polymerase inhibitors and/or NS3/4A protease-inhibitors. More-

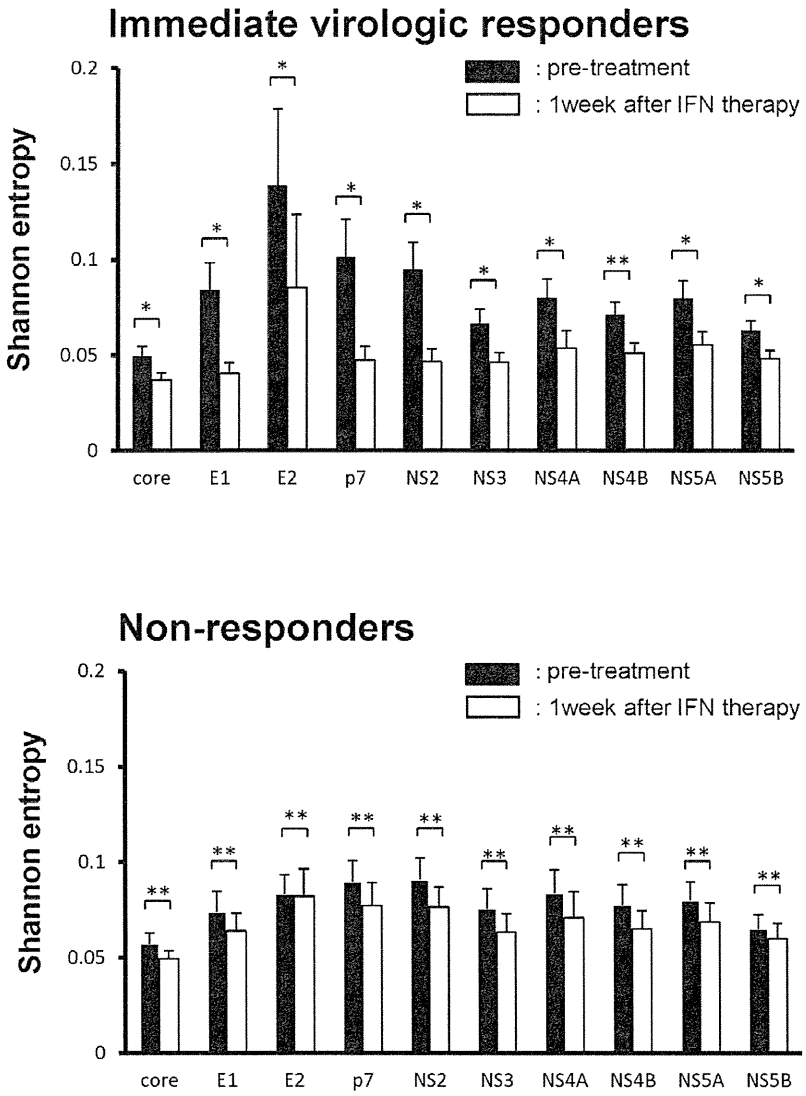

Figure 1. Changes in the genetic complexity of each HCV genomic region before and after the administration of peg-IFNα2b plus RBV. Shannon entropy values at baseline (black bar) and 1 week after initiation of treatment with peg-IFNα2b plus RBV (white bar) in 8 immediate virologic responders (A) and in 8 non-responders (B) are shown. * $p<0.05$, ** not significant. (Mean values ± SD; n = 8)
doi:10.1371/journal.pone.0024907.g001

over, 5 subjects (18.5%) harbored resistance to 6 antiviral drugs. Notably, 3 subjects harbored resistance to 8 of 9 antiviral drugs. There was no significant association between the frequency of drug-resistant mutations and the serum viral load ($r = 0.0678$) (Figure S1).

These findings indicate that drug-resistant HCV variants are present in a considerable proportion among the chronically HCV-infected, DAAs-naïve patients.

Discussion

Sequence heterogeneity, so-called quasispecies, is a common feature of RNA viruses, including HCV [22]. Previous studies of the viral genome with conventional Sanger sequencing methods revealed that HCV infection comprises a cloud of closely related sequence variants differing by as little as one nucleotide from a population average sequence [23]. A number of studies have aimed to clarify the significance of viral mutations in association with clinical features, including viral persistency and chronicity, degree of liver damage, response to treatment, and selection of mutants resistant to anti-viral therapy. The quasispecies nature of HCV, however, represents a major obstacle in determining the significance of the viral clone with specific sequence characteristics. Newly developed ultra-deep sequencing analysis allowed us to clarify the whole picture of viral quasispecies present in chronically HCV-infected patients. In the present study, ultra-deep sequencing determined a mean total of more than 10 million nucleotides of the viral genome in each specimen, representing more than 1000 clones infecting each patient, thus demonstrating the abundant genetic complexity of HCV.

It is well recognized that the HCV genome is heterogeneous at the intra-individual level [9,10]. The current ultra-deep sequenc-

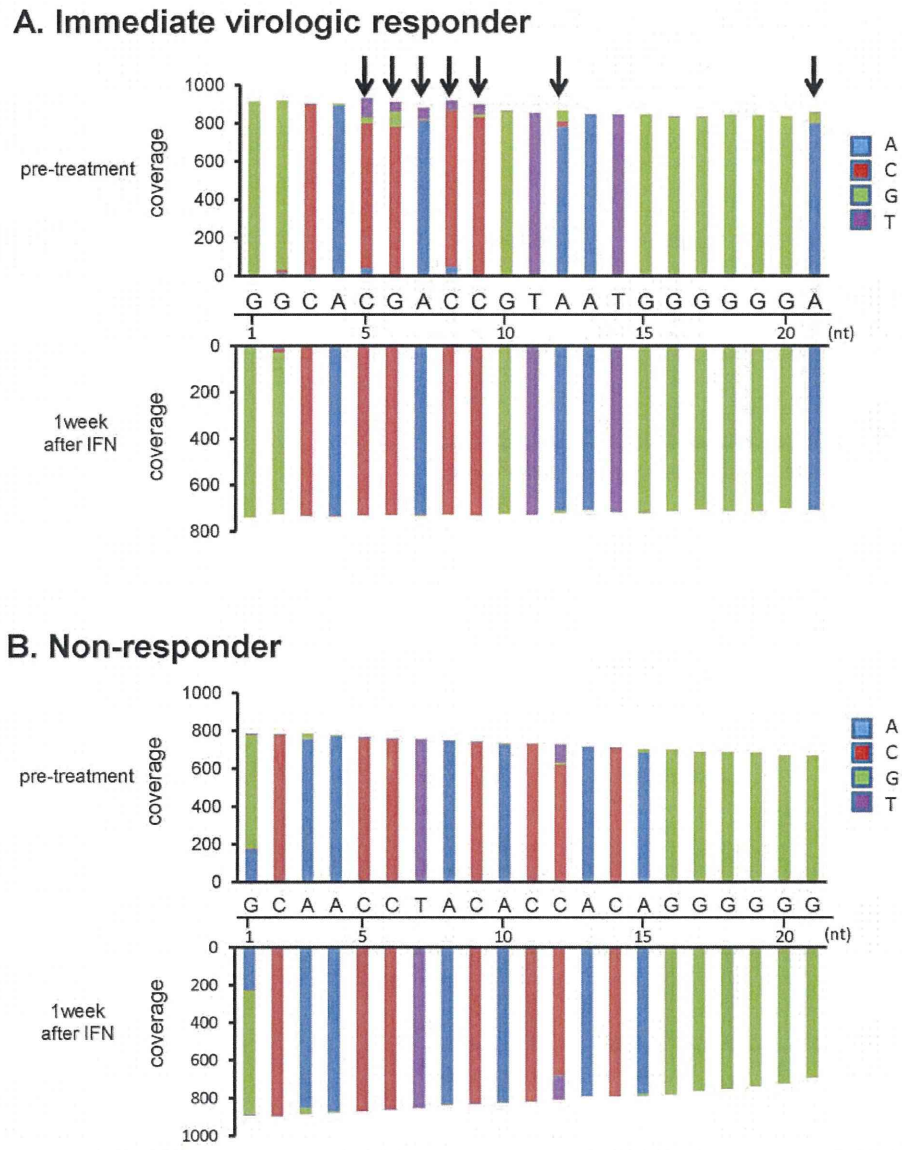

Figure 2. Ratio of mutated nucleotides in the HVR1 region before and after administration of peg-IFNα2b plus RBV therapy. Representative results of a immediate virologic responder (Patient#3) (**A**) and a non-responder (Patient#9) (**B**) are shown. The read numbers (coverage) at each nucleotide position of the HVR1 (from 1st nucleotide to 21st nucleotide in E2 region) at pre-treatment (upper graphs) and 1 week after initiating treatment with peg-IFNα2b plus RBV (lower graphs) are shown. Arrows indicate the nucleotide positions that showed the elimination of minor mutant clones after administration of peg-IFNα2b plus RBV.
doi:10.1371/journal.pone.0024907.g002

ing analyses revealed that the E2 region had the highest sequence heterogeneity, while the core region had the lowest sequence heterogeneity among the viral genomic regions encoding different functional viral proteins. More than 15% of nucleotides in the E2 region were mutated in all cases examined. These findings are consistent with previous conventional Sanger sequencing-based studies showing that HVR1 and HVR2 possess the highest sequence diversity among the HCV genomic regions [19] and that the highest values of mean Shannon entropy at the HCV 1a population level are in the E2 region [24].

Various mutations in the HCV genome are associated with the therapeutic response. For example, a number of mutations within a so-called IFNα sensitivity determining region of NS5A are closely associated with sensitivity to IFN-based anti-viral therapy [25,26]. A recent study also showed that amino acid substitution in the HCV core region could be a useful predictor of the virologic response to peg-IFNα plus RBV combination therapy [27]. Although the findings of these studies suggested that certain mutations in the representative HCV clone could predict treatment outcome, it is unknown whether the specific viral clone comprising those mutations directly displays sensitivity or resistance to anti-viral therapy. In the present study, sequential comparison of the HCV1b genome derived at baseline and at 1 week after the administration of peg-IFNα2b plus RBV demon-