Table 3. The prevalence of G1896A mutation in the pre-C region, and A1762T and G1764A mutations in the core-promoter region in the liver of patients chronically infected with HBV.

			Mutation Frequency					
	HBeAg/HBeAb	NA (duration of treatment)	G1896A (Pre C)		A1762T (CP)		G1764A (CP)	
Chronic-naive								
Liver #1	+/−	-	640/1652	(38.7)	1647/1941	(84.9)	1683/1979	(85.0)
Liver #2	+/−	-	9/596	(1.5)	682/687	(99.3)	683/689	(99.1)
Liver #3	+/−	-	273/672	(40.6)	767/769	(99.7)	757/760	(99.6)
Liver #4	+/−	-	204/701	(29.1)	610/625	(97.6)	602/621	(96.9)
Liver #5	+/−	-	27/152	(17.8)	249/250	(99.6)	245/248	(98.8)
Liver #6	+/−	-	228/621	(36.7)	727/729	(99.7)	743/744	(99.9)
Liver #7	−/+	-	740/1193	(62.0)	1908/1913	(99.7)	1888/1913	(98.7)
Liver #8	−/+	-	111/1892	(5.9)	2321/2325	(99.8)	2335/2339	(99.8)
Liver #9	−/+	-	10935/10944	(99.9)	12019/12032	(99.9)	12163/12170	(99.9)
Liver #10	−/+	-	4554/4593	(99.2)	1/5191	(0)	4/5188	(0.1)
Liver #11	−/+	-	811/921	(88.1)	1234/1236	(99.8)	1226/1228	(99.8)
Liver #12	−/+	-	93/1265	(7.4)	1234/1234	(100)	1228/1229	(99.9)
Liver #13	−/+	-	83/877	(9.5)	1465/1529	(95.8)	1485/1549	(95.9)
Liver #14	−/+	-	0/717	(0)	1078/1410	(76.5)	1089/1414	(77.0)
Chronic-NA								
Liver #15	−/+	LAM (156w)	0/390	(0)	441/453	(97.4)	435/448	(97.1)
Liver #16	−/+	ETV (1w)	0/1399	(0)	1624/1632	(99.5)	1625/1630	(99.7)
Liver #17	−/+	LAM (144w)	345/816	(42.3)	988/991	(99.7)	994/994	(100)
Liver #18	−/+	LAM (98w)	2/3963	(0.1)	1015/1188	(85.4)	1190/1194	(99.7)
Liver #19	−/+	LAM (11w)	48/4214	(1.1)	3438/3456	(99.5)	3446/3462	(99.5)

Values in parenthesis show mutation frequency (%): the ratio of total mutant clones to total aligned coverage at each nucleotide sites.
NA: nucleotide analogue, pre C: precore, CP: core promoter, LAM: lamivudine, ETV: entecavir.
doi:10.1371/journal.pone.0035052.t003

The prevalence of the 9 drug-resistant mutations detected by ultra-deep sequencing in 14 chronic-naïve cases ranged from 0.3% to 30.0%, indicating that the proportion of resistant mutations substantially differed in each case. The most commonly detected mutation was M204VI (9 cases) and M250VI (11 cases), which were resistant to lamivudine and entecavir, and entecavir, respectively. Other mutations resistant to adefovir were detected in 7 (50.0%) and 3 (21.4%) cases at A181TV and N236T, respectively (Table 4).

Nine (64.2%) chronic-naïve cases possessed the M204VI mutants in their liver tissues and the proportion of mutant clones among the totally infected viruses ranged from 0.3% to 1.1% among the M204VI mutant-positive patients. In chronic-NA cases, 4 of 5 (80.0%) liver tissues harbored the M204VI mutants with the proportion among the totally infected viruses ranging from 0.4% to 18.7% (Table 4), while the mean serum HBV DNA was suppressed below 2.6 log copies/ml (Table 1). These results suggest that the mutant HBV clones comprising various drug-resistant mutations could latently exist even in the liver of NA treatment-naïve cases.

Expansion of drug-resistant HBV clones harboring M240VI mutations in response to NA administration

To clarify the risk of latent expansion of drug-resistant mutations due to NA treatment, we next examined the early dynamic changes of the prevalence of M204VI mutants in the serum of treatment-naïve patients in response to entecavir treatment. Ultra-deep sequencing provided a mean 40,791- and 38,823-fold coverage of readings, which were mapped to the M204VI nucleotide position at the YMDD sites of each reference sequence in patients before and after entecavir treatment.

Five of 14 (35.7%) patients harbored the M204VI mutations prior to entecavir treatment. Although the serum HBV DNA levels were significantly reduced in response to entecavir in all cases, the M204VI mutant clones were detected in 9 cases (64.3%) after entecavir administration (Table 5). Notably, one patient (Serum #3) who harbored the M240VI mutant clones at baseline had a relatively large expansion of drug-resistant clones among the total viral population in a time-dependent manner in response to entecavir treatment (Table 5). Similarly, M240VI mutant clones became detectable after entecavir administration in four patients (Serum #1, #7, #12, #13) that harbored no resistant mutants at baseline (Table 5). We found no correlation between the degree of the increase in the relative prevalence of M204VI mutant clones and that of the reduction in serum HBV DNA levels. Although only a limited number of patients exhibited a substantial increase in M204VI mutant clones after administration of anti-viral therapy, our findings might suggest that entecavir treatment latently causes selective survival of drug-resistant mutants in treatment naïve patients with chronic HBV infection.

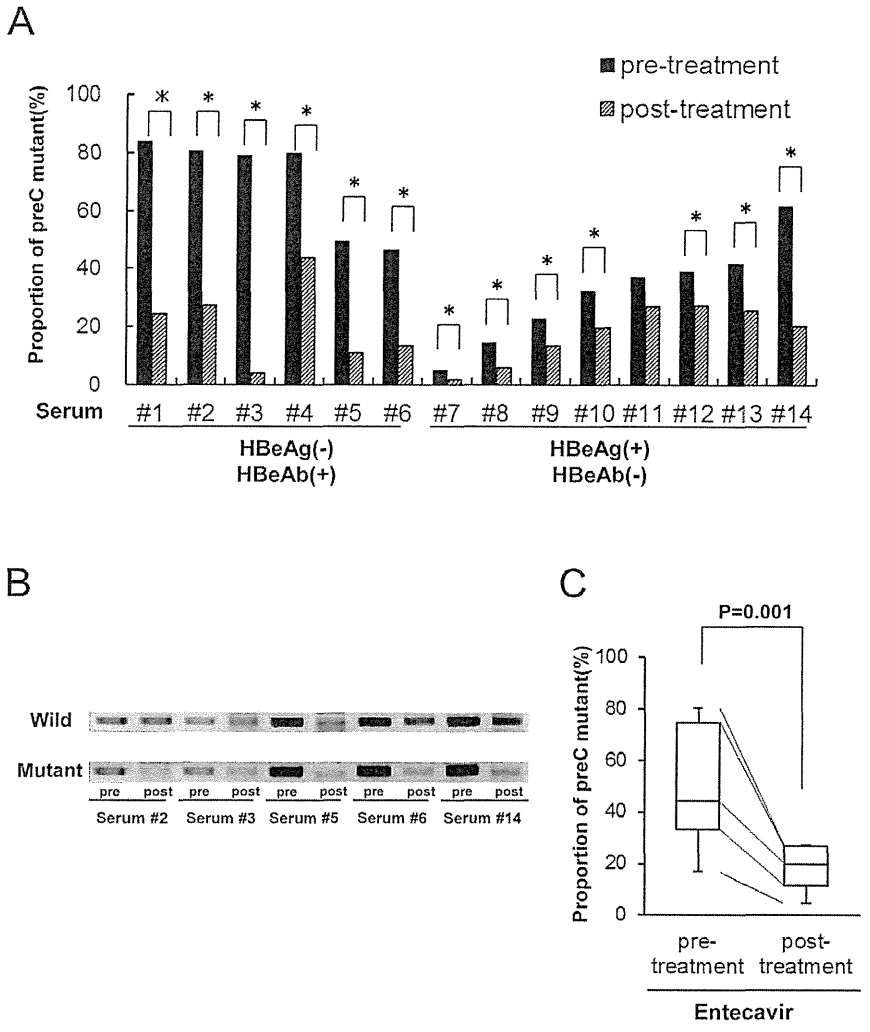

Figure 2. The reduction in the relative proportion of the G1896A pre-C mutant clones after entecavir administration. (A) The relative proportion of the G1896A pre-C mutant was determined in the serum of treatment-naïve patients pre- and post-entecavir administration using quantitative real-time PCR. Serum #1~6 were HBeAg-negative and HBeAb-positive, and Serum #7~14 were HBeAg-positive and HBeAb-negative before treatment. *: $p<0.05$ (B) Semiquantitative PCR analysis was performed using primers specific to the wild-type (upper panel) or G1896A pre-C mutant (lower panel) pre- and post-entecavir administration. A representative result from 5 cases is shown. (C) The relative proportion of the G1896A pre-C mutant was compared in 14 treatment-naïve patients between pre- and post-entecavir administration.
doi:10.1371/journal.pone.0035052.g002

Discussion

Direct population sequencing is the most common method for detecting viral mutations [29]. Conventional sequencing techniques, however, are not efficient for evaluating large amounts of genetic information of the viruses. Newly developed ultra-deep sequencing technology have revolutionized genomic analyses, allowing for studies of the dynamics of viral quasispecies as well as rare genetic variants of the viruses that cannot be detected using standard direct population sequencing techniques [30,31]. The sensitivity of ultra-deep sequencing analysis is primarily limited by errors introduced during PCR amplification and the sequencing reaction, thus it is a challenge to distinguish rare variants from sequencing artifacts. In the present study, we optimized the ultra-deep sequencing with a multiplex-tagging method and reproducibly detected variants within HBV quasispecies that were as rare as 0.3%. Based on this ultra-deep sequencing platform, we determined the abundant genetic heterogeneity of HBV at the intra- and inter-individual levels.

Because of its ability to handle abundant viral genome information, ultra-deep sequencing allowed us to evaluate low-abundant virus variants of patients with chronic HBV infection in detail. It is widely accepted that HBe seroconversion is highly associated with the emergence of G1896A pre-C and/or A1762T and G1764A core promoter mutant clones [7–9]. Unexpectedly, however, our results showed a diverse range of G1896A frequency (0–99.9%) in HBeAg-negative subjects and a high prevalence of core promoter mutations, irrespective of HBe serostatus. Consistent with our observation, previous studies utilizing conventional sequencing methods reported that the frequency of the G1896A pre-C mutant ranged from 12% to 85% [32]. All but one patient (Liver #10) showing a predominance of A1762T and G1764A were infected with genotype C, while patient#10 was infected with genotype B. Because A1762T and G1764A are reported to be significantly more frequent in genotype C [33], the difference in the prevalence of A1762T and G1764A in our study might be a reflection of the viral HBV genotype rather than HBe serostatus. Further investigation of the actual prevalence of these mutations

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.
doi:10.1371/journal.pone.0035052.t004

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.
doi:10.1371/journal.pone.0035052.t005

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)

Acknowledgments

We thank Prof. G. Tsujimoto, Dr. F. Sato, Dr. Y. Matsumoto, Dr. Y. Endo, Dr. A Takai, Ms. Y. Nakagawa, Ms. K. Fujii and Ms. C. Hirano for ultra-deep sequencing analysis.

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.

References

1. Dienstag JL (2008) Hepatitis B virus infection. N Engl J Med 359: 1486–1500.
2. Lok AS, McMahon BJ (2007) Chronic hepatitis B. Hepatology 45: 507–539.
3. Chang KM (2010) Hepatitis B immunology for clinicians. Clin Liver Dis 14: 409–424.
4. Murray JM, Purcell RH, Wieland SF (2006) The half-life of hepatitis B virions. Hepatology 44: 1117–1121.
5. Ngui SL, Teo CG (1997) Hepatitis B virus genomic heterogeneity: variation between quasispecies may confound molecular epidemiological analyses of transmission incidents. J Viral Hepat 4: 309–315.
6. Hollinger FB (2007) Hepatitis B virus genetic diversity and its impact on diagnostic assays. J Viral Hepat 14 Suppl 1: 11–15.
7. Akahane Y, Yamanaka T, Suzuki H, Sugai Y, Tsuda F, et al. (1990) Chronic active hepatitis with hepatitis B virus DNA and antibody against e antigen in the serum. Disturbed synthesis and secretion of e antigen from hepatocytes due to a point mutation in the precore region. Gastroenterology 99: 1113–1119.
8. Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, et al. (1990) Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J Virol 64: 1298–1303.
9. Kramvis A, Kew MC (1999) The core promoter of hepatitis B virus. J Viral Hepat 6: 415–427.
10. Carman WF, Fagan EA, Hadziyannis S, Karayiannis P, Tassopoulos NC, et al. (1991) Association of a precore genomic variant of hepatitis B virus with fulminant hepatitis. Hepatology 14: 219–222.
11. Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M (1991) Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 324: 1699–1704.
12. Domingo E, Gomez J (2007) Quasispecies and its impact on viral hepatitis. Virus Res. Netherlands. pp 131–150.
13. Fishman SL, Branch AD (2009) The quasispecies nature and biological implications of the hepatitis C virus. Infect Genet Evol 9: 1158–1167.
14. Kwon H, Lok AS (2011) Hepatitis B therapy. Nat Rev Gastroenterol Hepatol.
15. Dienstag JL (2009) Benefits and risks of nucleoside analog therapy for hepatitis B. Hepatology 49: S112–121.
16. Ghany MG, Doo EC (2009) Antiviral resistance and hepatitis B therapy. Hepatology 49: S174–184.
17. Zoulim F, Locarnini S (2009) Hepatitis B virus resistance to nucleos(t)ide analogues. Gastroenterology 137: 1593–1608.e1591–1592.
18. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, et al. (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437: 376–380.
19. Nasu A, Marusawa H, Ueda Y, Nishijima N, Takahashi K, et al. (2011) Genetic heterogeneity of hepatitis C virus in association with antiviral therapy determined by ultra-deep sequencing. PLoS One 6: e24907.
20. Margeridon-Thermet S, Shulman NS, Ahmed A, Shahriar R, Liu T, et al. (2009) Ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naive patients. J Infect Dis 199: 1275–1285.
21. Solmone M, Vincenti D, Prosperi MC, Bruselles A, Ippolito G, et al. (2009) Use of massively parallel ultradeep pyrosequencing to characterize the genetic diversity of hepatitis B virus in drug-resistant and drug-naive patients and to detect minor variants in reverse transcriptase and hepatitis B S antigen. J Virol 83: 1718–1726.
22. Han Y, Huang LH, Liu CM, Yang S, Li J, et al. (2009) Characterization of hepatitis B virus reverse transcriptase sequences in Chinese treatment naive patients. J Gastroenterol Hepatol 24: 1417–1423.
23. Ikeda K, Marusawa H, Osaki Y, Nakamura T, Kitajima N, et al. (2007) Antibody to hepatitis B core antigen and risk for hepatitis C-related hepatocellular carcinoma: a prospective study. Ann Intern Med 146: 649–656.
24. Ruike Y, Imanaka Y, Sato F, Shimizu K, Tsujimoto G (2010) Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. BMC Genomics 11: 137.
25. Louvel S, Battegay M, Vernazza P, Bregenzer T, Klimkait T, et al. (2008) Detection of drug-resistant HIV minorities in clinical specimens and therapy failure. HIV Med 9: 133–141.
26. Ntziora F, Paraskevis D, Haida C, Magiorkinis E, Manesis E, et al. (2009) Quantitative detection of the M204V hepatitis B virus minor variants by amplification refractory mutation system real-time PCR combined with molecular beacon technology. J Clin Microbiol 47: 2544–2550.
27. Matsumoto Y, Marusawa H, Kinoshita K, Endo Y, Kou T, et al. (2007) Helicobacter pylori infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. Nat Med 13: 470–476.
28. Marusawa H, Matsuzawa S, Welsh K, Zou H, Armstrong R, et al. (2003) HBXIP functions as a cofactor of survivin in apoptosis suppression. EMBO J 22: 2729–2740.
29. Lok AS, Zoulim F, Locarnini S, Bartholomeusz A, Ghany MG, et al. (2007) Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. Hepatology 46: 254–265.
30. Hedskog C, Mild M, Jernberg J, Sherwood E, Bratt G, et al. (2010) Dynamics of HIV-1 quasispecies during antiviral treatment dissected using ultra-deep pyrosequencing. PLoS One 5: e11345.
31. Rozera G, Abbate I, Bruselles A, Vlassi C, D'Offizi G, et al. (2009) Massively parallel pyrosequencing highlights minority variants in the HIV-1 env quasispecies deriving from lymphomonocyte sub-populations. Retrovirology 6: 15.
32. Chowdhury A, Santra A, Chakravorty R, Banerji A, Pal S, et al. (2005) Community-based epidemiology of hepatitis B virus infection in West Bengal, India: prevalence of hepatitis B e antigen-negative infection and associated viral variants. J Gastroenterol Hepatol 20: 1712–1720.
33. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, et al. (2001) A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. Hepatology 33: 218–223.
34. Yu JW, Sun LJ, Yan BZ, Kang P, Zhao YH (2011) Lamivudine treatment is associated with improved survival in fulminant hepatitis B. Liver Int 31: 499–506.
35. Lisotti A, Azzaroli F, Buonfiglioli F, Montagnani M, Alessandrelli F, et al. (2008) Lamivudine treatment for severe acute HBV hepatitis. Int J Med Sci 5: 309–312.
36. Simen BB, Simons JF, Hullsiek KH, Novak RM, Macarthur RD, et al. (2009) Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. J Infect Dis 199: 693–701.
37. Lataillade M, Chiarella J, Yang R, Schnittman S, Wirtz V, et al. (2010) Prevalence and clinical significance of HIV drug resistance mutations by ultra-deep sequencing in antiretroviral-naïve subjects in the CASTLE study. PLoS One 5: e10952.

OPEN ACCESS Freely available online

PLOS ONE

Pretransplant Serum Hepatitis C Virus RNA Levels Predict Response to Antiviral Treatment after Living Donor Liver Transplantation

Yoshihide Ueda[1]*, Toshimi Kaido[2], Yasuhiro Ogura[2], Kohei Ogawa[2], Atsushi Yoshizawa[2], Koichiro Hata[2], Yasuhiro Fujimoto[2], Aya Miyagawa-Hayashino[3], Hironori Haga[3], Hiroyuki Marusawa[1], Satoshi Teramukai[4], Shinji Uemoto[2], Tsutomu Chiba[1]

1 Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **2** Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **3** Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Japan, **4** Division of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital, Kyoto, Japan

Abstract

Background: Given the limited efficacy and high adverse event rate associated with treatment of recurrent hepatitis C after liver transplantation, an individualized treatment strategy should be considered. The aim of this study was to identify predictors of response to antiviral therapy for hepatitis C after living donor liver transplantation (LDLT) and to study the associated adverse events.

Methods: A retrospective chart review was performed on 125 hepatitis C virus (HCV)-positive LDLT recipients who received interferon plus ribavirin and/or peginterferon plus ribavirin therapy at Kyoto University between January 2001 and June 2011.

Results: Serum HCV RNA reached undetectable levels within 48 weeks in 77 (62%) of 125 patients, and these patients were defined as showing virological response (VR). Of 117 patients, 50 (43%) achieved sustained VR (SVR). Predictive factors associated with both VR and SVR by univariate analysis included low pretransplant serum HCV RNA levels, a non-1 HCV genotype, and low pretreatment serum HCV RNA levels. In addition, LDLT from ABO-mismatched donors was significantly associated with VR, and white cell and neutrophil counts before interferon therapy were associated with SVR. Multivariate analysis showed that 2 variables–pretransplant serum HCV RNA level less than 500 kIU/mL and a non-1 HCV genotype–remained in models of both VR and SVR and that an ABO mismatch was associated with VR. No variables with a significant effect on treatment withdrawal were found.

Conclusions: Virological response to antiviral therapy in patients with hepatitis C recurring after LDLT can be predicted prior to transplant, based on pretransplant serum HCV-RNA levels and HCV genotype. LDLT from ABO-mismatched donors may contribute to more efficacious interferon therapy.

Trial Registration: UMIN-CTR UMIN000003286.

Citation: Ueda Y, Kaido T, Ogura Y, Ogawa K, Yoshizawa A, et al. (2013) Pretransplant Serum Hepatitis C Virus RNA Levels Predict Response to Antiviral Treatment after Living Donor Liver Transplantation. PLoS ONE 8(3): e58380. doi:10.1371/journal.pone.0058380

Editor: Hak Hotta, Kobe University, Japan

Received August 30, 2012; **Accepted** February 4, 2013; **Published** March 7, 2013

Funding: This work was supported by Japan Society for the Promotion of Science (JSPS) Grants-in-aid for Scientific Research 21229009 and 23590972, and Health and Labour Sciences Research Grants for Research on Intractable Diseases, and Research on Hepatitis from the Ministry of Health, Labour and Welfare, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: yueda@kuhp.kyoto-u.ac.jp

Introduction

Hepatitis C virus (HCV) infection, leading to liver cirrhosis and hepatocellular carcinoma, is the leading indications for liver transplantation in Japan, the United States, and Western Europe. However, almost all patients who undergo liver transplantation for HCV-related liver disease develop recurrent viral infection, and 70–90% of patients suffer from histologically proven recurrent hepatitis [1,2,3,4,5,6]. The progression of recurrent hepatitis C is often accelerated and, without appropriate antiviral therapy, 10–25% of patients develop cirrhosis within 5 years after transplantation, resulting in poorer prognoses for HCV-positive recipients than HCV-negative recipients [7]. To prevent the progression of hepatitis C after liver transplantation, interferon-based combination therapy is commonly administered [8,9]. However, its efficacy in liver transplant recipients is limited, with the mean sustained virological response (SVR) rate among patients with recurrent hepatitis C after liver transplantation being only 30% (range, 8–50%) [10]. One of the reasons for the low SVR rate is the high rate of treatment withdrawal. Several severe adverse events have been reported in transplant recipients after interferon therapy,

including chronic rejection and *de novo* autoimmune hepatitis [11,12,13].

To improve the efficacy of anti-HCV treatment in patients with hepatitis C after liver transplantation, an individualized treatment strategy based on efficacy prediction and adverse events should be attempted. In several studies, an analysis of predictors associated with SVR was conducted in patients with recurrent hepatitis C after deceased donor liver transplantation (DDLT) [10,14,15,16,17,18,19,20]. In these studies, variables most frequently associated with SVR were early virological response (EVR) at 3 months of therapy, HCV genotype 2, adherence to therapy, and baseline viremia [14,15,16,17,18,19,20]. Of these factors, EVR and adherence to therapy can only be recognized after the initiation of treatment. However, to enable decisions on treatment indications and strategy, predictors of response that are available before initiation of therapy are more valuable. Thus, an individualized treatment strategy could be based on the identification of baseline predictive factors before interferon therapy. Moreover, no study of factors predictive of response to the interferon therapy in patients with recurrent hepatitis C after living donor liver transplantation (LDLT) has been reported so far. Characteristics specific to LDLT, including blood-relative donors, post-transplant liver regeneration, and ABO-incompatible liver transplantation, might cause the antiviral effects of interferon therapy in these patients to differ from those who received DDLT.

The direct-acting antiviral agents telaprevir and boceprevir recently became available for clinical use. The results of clinical trials of these agents in combination with peginterferon plus ribavirin in nontransplant patients with HCV were promising [21,22,23,24]. SVR rates to telaprevir-based combination therapy were significantly higher than those to the peginterferon-ribavirin combination. The efficacy in the patients who had suffered a relapse after a previous treatment by peginterferon plus ribavirin was especially striking [21,24]. The SVR rate to telaprevir based-therapy in patients who had a previous relapse was more than 80%, while that in patients who had no response to previous treatment was around 30% [24]. These results suggest that patients who show a virological response (VR) to peginterferon plus ribavirin are expected to achieve SVR after telaprevir-based therapy. Therefore, identification of factors predictive of virological response to peginterferon plus ribavirin should also prove useful when making the clinical decision about telaprevir usage. In liver transplant recipients, the use of telaprevir and boceprevir poses risks because of their inhibitory action on the enzyme cytochrome P450 3A, responsible for the metabolism of both tacrolimus and cyclosporine. In fact, the phase I study of telaprevir in healthy individuals revealed that it significantly increased the blood concentrations of both tacrolimus and cyclosporine [25]. Therefore, the selection of the patients for whom telaprevir is prescribed is especially important in liver transplant recipients.

Recently, a polymorphism in the interleukin-28B (IL28B) gene region, encoding interferon-lambda 3, was identified as a strong predictive factor for response to antiviral treatment in nontransplant patients with hepatitis C [26,27,28]. In post-transplant patients, the IL28B polymorphism in both recipients and donors was shown to be associated with response to antiviral treatment [29,30]. In addition, HCV-RNA mutations, including those affecting amino acid (aa) residues 70 and 91 in the core region of HCV and those in the interferon sensitivity determining region (ISDR) in nonstructural protein 5A (NS5A), were also demonstrated to be predictors of response to interferon therapy in transplant recipients, as well as in nontransplant settings [31,32,33]. These factors could be used to predict response to antiviral therapy, but these are presently not part of a routine clinical examination and require special techniques not covered by health insurance. Moreover, probing individual genetic information poses potential ethical issues.

The aims of this study were, therefore, to identify noninvasively obtained regular baseline factors associated with VR, SVR, and treatment withdrawal, in order to elucidate the factors associated purely with response to interferon therapy, to identify the valuables related to final outcomes, and to clarify the factors associated with adverse events.

Methods

A retrospective chart review was performed for all HCV-positive liver transplant patients who received antiviral therapy with standard interferon and/or pegylated interferon in combination with ribavirin after liver transplantation at Kyoto University between January 2001 and June 2011.

Patients

Between March 1999 and June 2011, 214 HCV-positive recipients underwent LDLT at Kyoto University. Of these, 157 patients were followed up for more than 6 months after LDLT in our hospital. Anti-viral therapy was administered to 125 of the 157 patients with recurrent hepatitis C between January 2001 and June 2011. The remaining 32 patients did not receive anti-viral therapy for various reasons: serum HCV-RNA negative after LDLT (n = 4), no histological hepatitis C recurrence in the follow-up period (n = 13), no fibrosis seen by liver histology (n = 8), and ongoing treatment for the other complications (n = 7). HCV RNA concentrations and histological evidence were used to diagnose patients with recurrent hepatitis C after LDLT. These patients were given combination therapies with interferon plus ribavirin and/or peginterferon plus ribavirin at Kyoto University between January 2001 and June 2011. The study protocol was approved by the Ethics Committee at Kyoto University and performed in compliance with the Helsinki Declaration. Written informed consent for participation in this study was not obtained, because this study is an observational study without use of human specimen. Our institutional review board waived the need for written informed consent from the participants of the initial study.

Treatment Protocol and Definition of Responses to Treatment

Between January 2001 and April 2004, patients with recurrent hepatitis C after LDLT received treatment with interferon-α-2b (3 or 6 mega units, 3 times/week) plus ribavirin (400–800 mg/day orally), for the first 6 months. This was followed by interferon monotherapy for 6 months [34]. Forty patients received this treatment. Of the 40 patients, 14 patients achieved SVR and 9 withdrew from the treatment protocol. The remaining 17 patients, including 2 who relapsed and 15 nonresponders were retreated by the following protocol with peginterferon and rebavirin. Between May 2004 and June 2011, patients received combination therapy with peginterferon-α-2b (1.5 μg/kg) plus ribavirin (400–800 mg/day orally) [35]. Patients who acquired a negative serum HCV RNA status within 12 months after treatment initiation continued to receive the treatment for an additional 12 months before treatment termination. Total 102 patients, including 17 patients who had previously treated with standard interferon plus ribavirin and did not achieve SVR, were treated with this treatment protocol. Patients who were negative for serum HCV RNA for more than 6 months after completion of interferon therapy were defined as having achieved SVR. If serum HCV RNA was positive after 12 months of treatment, therapy was discontinued or

switched to maintenance therapy with low-dose peginterferon [36], and the patient was classified as having shown no response. Treatment was discontinued in patients with severe adverse events. Additionally, peginterferon treatment was discontinued when neutrophil and platelet counts fell below 500/μL and 30000/μL, respectively, and ribavirin was discontinued when hemoglobin levels fell below 8 g/dL.

We studied the final outcomes of the treatment with peginterferon plus ribavirin (n = 102) and with standard interferon plus ribavirin (n = 23).

Histological Assessment

Liver biopsies were performed when patients' alanine aminotransferase (ALT) levels were more than twice the normal upper limit, or at yearly intervals, with informed consent. Biopsy specimens were evaluated by 2 pathologists (H.H. and A.M-H.) with extensive experience in the pathology of liver transplantation. Necroinflammatory activity (A0–A3) and fibrosis stage (F0–F4) were assessed using METAVIR scores [37,38]. Activity was graded as A0 (no activity), A1 (mild activity), A2 (moderate activity), or A3 (severe activity); Fibrosis was staged as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis), or F4 (cirrhosis).

Immunosuppression

Tacrolimus and low-dose steroid therapy were administered to induce immunosuppression in most patients [34]. Four patients received cyclosporine microemulsions instead of tacrolimus. Mycophenolate mofetil was administered to patients who experienced refractory rejection or required reduction of tacrolimus or cyclosporine doses due to adverse events. Patients who received ABO blood-type incompatible transplants were treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone [39].

Virological Assays

HCV genotype was determined using a genotyping system based on polymerase chain reaction (PCR) to amplify the core region using genotype-specific PCR primers [40]. Serum HCV RNA load was evaluated before LDLT, before interferon treatment, once a month during treatment, and 24 weeks after treatment, using PCR and an Amplicor HCV assay (Cobas Amplicor HCV Monitor, Roche Molecular Systems, Pleasanton, CA, USA) until April 2008, or a real-time PCR-based quantitation method for HCV (COBAS AmpliPrep/COBAS TaqMan HCV Test, Roche Molecular Systems, Pleasanton, CA, USA) from May 2008. Detection of amino acid substitutions in the HCV core region was performed using the method reported previously [31].

Statistical Analysis

To evaluate the association between the patient characteristics and the outcomes (VR, SVR, or withdrawal), the Wald test was performed based on a logistic regression model. Multivariate logistic regression analysis with backward variable selection was used to identify independent and significant predictors for the outcomes, and to estimate the odds ratio (OR) ant its 95% confidence interval (CI). A p-value of 0.05 was used for variable selection and was regarded as significant. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary NC).

Results

Patient Characteristics

This study included 125 HCV-infected liver transplant patients treated with standard interferon and/or pegylated interferon in combination with ribavirin for recurrent hepatitis C after LDLT. Of the 125 patients, 69 (55%) were male, and the median age was 57 years (range: 15–70) at the beginning of the therapy. Most patients were infected with HCV genotype 1b (n = 103, 82%). HCV genotypes of the remaining patients were 2a (n = 13), 2b (n = 5), 3a plus 3b (n = 1), not determined (n = 2), and not examined (n = 1). Median serum HCV RNA load was 410 kIU/mL (range: <0.5–5000<kIU/mL) before LDLT, and 3260 kIU/mL (range: 31–69000<kIU/mL) at the beginning of the interferon therapy after LDLT. The median donor age was 41 (range: 19–65) years. Seventy-two donors (58%) were male, and 86 (69%) were related to the recipients. The graft type was the right lobe in 109 patients (87%), and the left lobe in 16 patients (13%). The blood type combination was incompatible in 26 patients (21%). The median time to treatment initiation after LDLT was 9.0 months (1.1–85.3 months). Before treatment, the necroinflammatory activity was A1 or greater in all patients, and 104 patients (83%) had a fibrosis score of F1 or greater (METAVIR score). Tacrolimus-based immunosuppression was used in 116 patients (93%). Among patients receiving tacrolimus for immunosuppression, the mean serum trough level was 6.0 ng/mL (range: 2.0–12.7) at the initiation of interferon therapy. In addition to calcineurin inhibitors, mycophenolate mofetil and prednisolone were used at the initiation of the interferon treatment in 36 (29%) and 19 (15%) patients, respectively.

Efficacy of Interferon Therapy

Of the 125 patients who received interferon therapy, serum HCV RNA reached undetectable levels (less than 0.05 kIU/mL) within 48 weeks in 77 patients (62%) (Figure 1). These patients were defined as showing virological response (VR). Of the remaining 48 patients, 2 patients received treatment for less than 48 weeks, and 15 patients withdrew from the treatment protocol within 48 weeks because of worsening of liver function (n = 5), recurrent hepatocellular carcinoma (n = 2), ascites (n = 2), anemia (n = 1), leucopenia (n = 1), brain hemorrhage (n = 1), biliary complication (n = 1), sepsis (n = 1), or myocardial infarction (n = 1). The remaining 31 patients with detectable HCV RNA in the serum 48 weeks after the initiation of the treatment were placed in the non-VR group. All patients in the non-VR group received peginterferon plus ribavirin therapy, including 9 patients who had previously treated with standard interferon plus ribavirin and did not achieve SVR. Of the patients with VR, 11 discontinued the treatment protocol within 24 weeks after serum HCV-RNA became negative, and 6 patients are still under treatment. The reasons for discontinuation were biliary complications (n = 2), worsening of liver function (n = 2), general fatigue (n = 2), recurrent hepatocellular carcinoma (n = 1), leucopenia (n = 1), hemoptysis (n = 1), brain tumor (n = 1), and depression (n = 1). Of 60 patients who achieved VR and completed the treatment protocol, 50 achieved SVR and 10 relapsed. None of the non-VR patients achieved VR even after more than 48 weeks of treatment, and were classified as nonresponder (NR).

In summary, among the 117 patients in whom the final outcomes of the treatment could be evaluated, 50 patients (43%) achieved SVR, and the remaining 67 patients, including 10 who relapsed (9%), 31 NR (26%), and 26 withdrawals (22%), were classified as non-SVR.

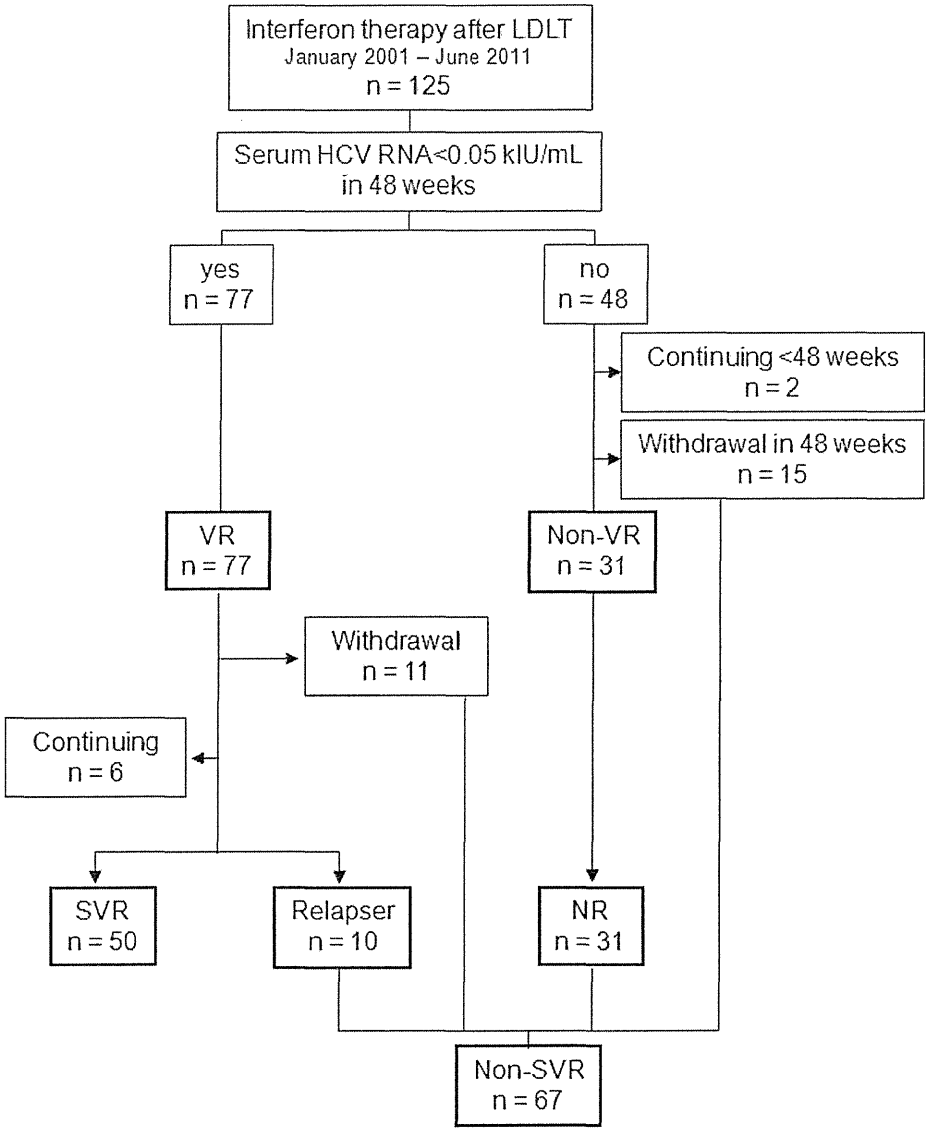

Figure 1. Flow diagram showing the outcome of interferon therapy in patients with recurrent hepatitis C after living donor liver transplantation (LDLT) and indicating the classification of patients in this study. N, number of patients; VR, virological response; SVR, sustained virological response; NR, nonresponder.
doi:10.1371/journal.pone.0058380.g001

Factors Predictive of Virological Response

Factors that could predict virological response were analyzed by comparing patients in the VR (n = 77) and non-VR (n = 31) groups (Table 1). Univariate analysis demonstrated that a low pretransplant serum HCV RNA level (less than 500 kIU/mL, $P<0.001$; and less than 1000 kIU/mL, $P<0.001$), an ABO-mismatched donor ($P=0.036$), HCV genotype (non-1, $P=0.001$), and a low pretreatment serum HCV RNA level (less than 5000 kIU/mL, $P=0.020$) were significantly associated with VR. There were no significant associations with any other variables, including donor factors. Multivariate analysis revealed that the 3 variables that retained a significant association in the model were a pretransplant serum HCV RNA level less than 500 kIU/mL [odds ratio (OR): 0.178, 95% confidence interval (CI): 0.054–0.535, $P=0.001$], a non-1 HCV genotype (OR: 0.087, 95% CI: 0.000–0.589, $P=0.008$), and an ABO-mismatched donor (OR: 5.492, 95% CI: 1.004–58.06, $P=0.049$) (Table 2). All 20 patients with a non-1 HCV genotype achieved VR, while VR rate in patients with the HCV genotype 1 was 65% (57 out of 88 patients). In the patients with HCV genotype 1, VR rate was 80% (36 of 45 patients) when pretransplant serum HCV-RNA level was less than 500 kIU/mL and 42% (15 of 36 patients) when it was 500 kIU/mL or more. Among 22 recipients from ABO-mismatched donors, 20 patients (91%) showed VR, while 57 (66%) out of 86 patients who underwent LDLT from an ABO-matched (identical and compatible) donor achieved VR.

Factors Predictive of SVR

The same variables were analyzed to clarify factors that predicted SVR by comparing patients in the SVR (n = 50) and non-SVR (n = 67) groups (Table 1). By univariate analysis, the same variables that had a significant association with VR were identified as significant predictive factors for SVR–low pretransplant serum HCV RNA levels (less than 100 kIU/mL, $P=0.028$;

Table 1. Baseline predictive factors before liver transplantation (pre-LT), at liver transplantation (at LT), and before interferon therapy (pre-IFN) associated with virorogical response (VR) and sustained VR (SVR): Univariate analysis.

		VR	non-VR	*P*	SVR	non-SVR	*P*
		n = 77	n = 31		n = 50	n = 67	
Age at LT (years)		55 (8–67)	56 (37–69)	0.462	54.5 (8–67)	56 (30–69)	0.212
Gender	Male	45 (74%)	16 (26%)	0.518	30 (46%)	35 (54%)	0.404
	Female	32 (68%)	15 (32%)		20 (38%)	32 (62%)	
HCC pre-LT	No	29 (71%)	12 (29%)	0.919	18 (43%)	24 (57%)	0.984
	Yes	48 (72%)	19 (28%)		32 (43%)	43 (57%)	
MELD pre-LT		15.5 (3–51)	15 (6–25)	0.403	16 (3–51)	15 (0–43)	0.616
Child-Pugh pre-LT	A/B	35 (74%)	12 (26%)	0.488	25 (49%)	26 (51%)	0.192
	C	41 (68%)	19 (32%)		24 (37%)	41 (63%)	
	unknown	1	0		1	0	
Serum HCV RNA pre-LT	<100 kIU/mL	16 (89%)	2 (11%)	0.063	11 (65%)	6 (35%)	0.028
	100 kIU/mL≤	52 (65%)	28 (35%)		31 (35%)	57 (65%)	
	unknown	9	1		8	4	
Serum HCV RNA pre-LT	<500 kIU/mL	50 (85%)	9 (15%)	<0.001	30 (55%)	25 (45%)	0.002
	500 kIU/mL≤	18 (46%)	21 (54%)		12 (24%)	38 (76%)	
	unknown	9	1		8	4	
Serum HCV RNA pre-LT	<1000 kIU/mL	56 (81%)	13 (19%)	<0.001	34 (49%)	36 (51%)	0.013
	1000 kIU/mL≤	12 (41%)	17 (59%)		8 (23%)	27 (77%)	
	unknown	9	1		8	4	
HCV genotype	Non-1	20 (100%)	0 (0%)	0.001	15 (79%)	4 (21%)	0.002
	1	57 (65%)	31 (35%)		35 (36%)	62 (64%)	
	unknown				0	1	
Donor age at LT (years)		42 (20–63)	38 (21–61)	0.504	43 (20–60)	38 (19–63)	0.748
Donor gender at LT	Male	41 (67%)	20 (33%)	0.287	27 (40%)	40 (60%)	0.538
	Female	36 (77%)	11 (23%)		23 (46%)	27 (54%)	
Sex mismatch	Match	28 (72%)	11 (28%)	0.932	18 (43%)	24 (57%)	0.984
	Mismatch	49 (71%)	20 (29%)		32 (43%)	43 (57%)	
ABO mismatch	Match	57 (66%)	29 (34%)	0.036	38 (40%)	56 (60%)	0.310
	Mismatch	20 (91%)	2 (9%)		12 (52%)	11 (48%)	
Relation of donor	Nonrelated	24 (73%)	9 (27%)	0.827	16 (44%)	20 (56%)	0.803
	Related	53 (71%)	22 (29%)		34 (42%)	47 (58%)	
Graft type	Left lobe	13 (81%)	3 (19%)	0.347	8 (62%)	5 (38%)	0.155
	Right lobe	64 (70%)	28 (30%)		42 (40%)	62 (60%)	
Splenectomy	No	38 (68%)	18 (32%)	0.413	25 (39%)	39 (61%)	0.378
	Yes	39 (75%)	13 (25%)		25 (47%)	28 (53%)	
Age pre-IFN (years)		57 (15–68)	57 (41–70)	0.494	56 (15–68)	57 (32–70)	0.200
Months from LT to therapy		9.2 (1.1–85.3)	8.9 (1.8–59.0)	0.846	9.0 (1.3–85.3)	9.0 (1.3–72.4)	0.879
Trough level for tacrolimus (ng/mL) pre-IFN		5.9 (2.0–10.9)	6.4 (3.3–10.6)	0.323	6.2 (2.2–9.5)	5.9 (2.0–12.7)	0.933
MMF pre-IFN	No	55 (71%)	23 (29%)	0.772	36 (43%)	48 (57%)	0.966
	Yes	22 (73%)	8 (27%)		14 (42%)	19 (58%)	
Prednisolone pre-IFN	No	64 (70%)	28 (30%)	0.347	41 (41%)	60 (59%)	0.245
	Yes	13 (81%)	3 (19%)		9 (56%)	7 (44%)	
Serum HCV RNA pre-IFN	<1000 kIU/mL	17 (89%)	2 (11%)	0.064	8 (38%)	13 (62%)	0.583
	1000 kIU/mL≤	58 (67%)	29 (33%)		42 (45%)	52 (55%)	
	unknown	2	0		0	2	
Serum HCV RNA pre-IFN	<5000 kIU/mL	52 (78%)	15 (22%)	0.020	36 (50%)	36 (50%)	0.030
	5000 kIU/mL≤	18 (55%)	15 (45%)		10 (28%)	26 (72%)	
	unknown	7	1		4	5	

Table 1. Cont.

		VR	non-VR	P	SVR	non-SVR	P
		n=77	n=31		n=50	n=67	
White cell count (102/mL)		51 (13–114)	49 (17–98)	0.135	49 (18–114)	48.5 (13–99)	0.049
Neutrophil count (102/mL)		26 (8–89)	22 (11–58)	0.127	26 (11–89)	23 (8–61)	0.044
Hemoglobin (g/dL)		12.0 (9.2–17.2)	12.0 (8.9–17.9)	0.638	12.0 (9.4–17.2)	11.8 (8.9–17.9)	0.157
Platelet count (104/mL)		21.7 (4.7–58.1)	15.1 (4.3–40.0)	0.153	20.3 (5.0–58.1)	15.8 (4.3–45.8)	0.165
AST (IU/L)		78 (19–352)	72 (25–464)	0.677	85 (21–352)	75 (24–547)	0.887
ALT (IU/L)		93 (18–395)	82 (21–392)	0.544	106 (22–395)	82 (18–597)	0.251
ALP (IU/L)		461 (199–1985)	433 (168–2977)	0.345	470 (204–1985)	470 (168–2977)	0.610
g-GTP (IU/L)		118.5 (15–1623)	114 (20–1827)	0.856	141 (15–1623)	115 (20–1827)	0.356
Bilirubin (mg/dL)		0.9 (0.3–11.0)	0.9 (0.3–10.4)	0.827	0.9 (0.4–11.0)	1.0 (0.3–13.7)	0.611
Activity grade pre-IFN	A1	54 (75%)	18 (25%)	0.448	35 (47%)	40 (53%)	0.517
	A2	22 (65%)	12 (35%)		14 (36%)	25 (64%)	
	A3	1 (50%)	1 (50%)		1 (33%)	2 (67%)	
Fibrosis stage pre-IFN	F0	9 (60%)	6 (40%)	0.446	6 (32%)	13 (68%)	0.530
	F1	54 (75%)	18 (25%)		34 (46%)	40 (54%)	
	F2/3	14 (67%)	7 (33%)		10 (42%)	14 (58%)	
Steatosis (5%<) pre-IFN	No	40 (69%)	18 (31%)	0.609	27 (42%)	38 (58%)	0.633
	Yes	36 (73%)	13 (27%)		23 (46%)	27 (54%)	
	unknown	1	0		0	2	
Cholestasis pre-IFN	No	58 (71%)	24 (29%)	0.903	38 (42%)	53 (58%)	0.577
	Yes	18 (72%)	7 (28%)		12 (48%)	13 (52%)	
	unknown	1	0		0	1	

NOTE. Qualitative variables are shown in number; and quantitative variables expressed as median (range). P-values are calculated by Wald test for logistic regression analysis.

LT, liver transplantation; HCC, hepatocellular carcinoma; MELD, model for end-stage liver disease; HCV, hepatitis C virus; MMF, mycophenolate mofetil; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; g-GTP, gamma-glutamyl transpeptidase.

doi:10.1371/journal.pone.0058380.t001

less than 500 kIU/mL, $P=0.002$; and less than 1000 kIU/mL, $P=0.013$), HCV genotype (non-1, $P=0.002$), and low pretreatment serum HCV RNA levels (less than 5000 kIU/mL, $P=0.030$). In addition, white cell count ($P=0.049$) and neutrophil count ($P=0.044$) before interferon therapy were significantly associated with SVR. Multivariate analysis showed that 2 variables were independently associated with SVR–a non-1 HCV genotype (OR: 0.182, 95% CI: 0.054–0.614, $P=0.006$), and pretransplant serum HCV RNA levels lower than 500 kIU/mL (OR: 0.310, 95% CI: 0.130–0.742, $P=0.009$) (Table 3). SVR rate among patients with a non-1 HCV genotype was 79% (15 of 19 patients) on average, 83% (10 of 12 patients) when pretransplant serum HCV-RNA level was less than 500 kIU/mL, and 50% (2 of 4 patients) when it was 500 kIU/mL or more. In patients with HCV genotype 1, SVR rate was 36% (35 of 97 patients) on average, 47% (20 of 43 patients) when pretransplant serum HCV-RNA level was less than 500 kIU/mL, and 22% (10 of 45 patients) when it was 500 kIU/mL or more.

Amino Acid Substitutions in Core Region of HCV

To determine the viral factors that predicted VR and SVR in patients infected with HCV genotype 1b, association of aa substitutions at aa 70 of arginine or glutamine/histidine and aa

Table 2. Predictive factors associated with virological response (VR): Multivariate analysis.

		Odds Ratio	95% confidence intervals	P-value
Serum HCV RNA pre-LT	<500 kIU/mL	1	-	-
	500 kIU/mL≤	0.178	0.054–0.535	0.001
HCV genotype	Non-1	1	-	-
	1	0.087	0.000–0.589	0.008
ABO mismatch	Match	1	-	-
	Mismatch	5.492	1.004-58.06	0.049

HCV, hepatitis C virus; LT, liver transplantation.

doi:10.1371/journal.pone.0058380.t002

Table 3. Predictive factors associated with sustained virological response (SVR): Multivariate analysis.

		Odds Ratio	95% confidence intervals	P-value
HCV genotype	Non-1	1	-	-
	1	0.182	0.054–0.614	0.006
Serum HCV RNA pre-LT	<500 kIU/mL	1	-	-
	500 kIU/mL≤	0.310	0.130–0.742	0.009

HCV, hepatitis C virus; LT, liver transplantation.
doi:10.1371/journal.pone.0058380.t003

91 of leucine or methionine with VR and SVR were analyzed in 40 patients, whose pre-treatment sera were stored (Table 4). As a result, substitutions of both aa 70 and aa 91 were not significantly associated with VR and SVR.

Predictors of Withdrawal from Therapy

Predictive factors for withdrawal from the treatment protocol were evaluated by comparing 26 patients who withdrew from the treatment protocol and the patients who completed the treatment including patients with SVR, patients who relapsed, and NR. None of the variables analyzed had a significant effect on withdrawal (Data not shown).

Discussion

In this study, we identified 2 independent predictors of SVR in patients with recurrent hepatitis C after LDLT by multivariate analysis: A non-1 HCV genotype and pretransplant serum HCV-RNA levels lower than 500 kIU/mL. The same factors were identified as predictors for VR, which purely indicates response to interferon therapy, by excluding the influences of the premature termination of the therapy and virological relapse after termination of the treatment. In addition, an ABO-incompatible LDLT was identified as an independent variable predicting VR.

In non-transplant settings, pretreatment predictors of response to interferon therapy have been analyzed in many studies, and the viral genotype and pretreatment viral load have been almost invariably shown to be 2 major predictors of SVR [41,42,43,44]. SVR rates were higher in patients infected with a non-1 HCV genotype and in those with a low pretreatment viral load. These 2 factors have been also identified in several reports [16,17,18,19] as factors predicting SVR in patients with recurrent hepatitis C after DDLT. In the present study, a non-1 HCV genotype was again identified as an independent predictive factor for both VR and SVR in patients with recurrent hepatitis C after LDLT by multivariate analysis. A pretreatment viral load <5000 kIU/mL was also a significant predictive factor by univariate analysis, but it was not an independently associated variable by multivariate analysis. On the other hand, pretransplant viral load was identified as an independent variable predictive of both VR and SVR by multivariate analysis.

While reports of factors that can control viral load exist, the mechanism by which serum HCV-RNA levels are regulated has not yet been completely clarified. A correlation between mutations in the ISDR sequence in the NS5A region of the HCV genome and serum HCV RNA levels has been reported. We did not analyze this viral factor in the current study; however, it is possible that the HCV genome sequence determines both pretransplant viremia and response to interferon therapy. The host polymorphism in IL28B, which was identified as a strong predictor of virological response to interferon therapy in patients with hepatitis C, was recently reported to be associated with baseline viral load [26,45]. The allele associated with a better treatment response is associated with a higher baseline viral load. This finding does not correspond with our results showing that a low HCV load predicts a better response to treatment. We speculate that the balance between host immunity and HCV replication regulates the serum HCV load, and that this balance also determines VR. As pretreatment viral load in post-transplant patients is influenced by immunosuppressive agents, the original host-virus balance

Table 4. Association of amino acid substitutions in the core region with virorogical response (VR) and sustained VR (SVR) in 40 patients infected with HCV genotype 1b: Univariate analysis.

		VR	non-VR	*P*	SVR	non-SVR	*P*
		n=22	n=13		n=14	n=24	
Core aa 70	Arg	9 (75%)	3 (25%)	0.289	7 (50%)	7 (50%)	0.204
	Gln/His	13 (57%)	10 (43%)		7 (29%)	17 (71%)	
Core aa 91	Leu	14 (64%)	8 (36%)	0.902	9 (38%)	15 (63%)	0.912
	Met	8 (62%)	5 (38%)		5 (36%)	9 (64%)	
Core aa 70 and 91	70 Arg and 91 Leu	6 (67%)	3 (33%)	0.784	5 (50%)	5 (50%)	0.320
	Others	16 (62%)	10 (38%)		9 (32%)	19 (68%)	
Core aa 70 and 91	70 Gln/His and 91 Met	5 (50%)	5 (50%)	0.324	3 (30%)	7 (70%)	0.603
	Others	17 (68%)	8 (32%)		11 (39%)	17 (61%)	

NOTE. Data are shown in number. P-values are calculated by Wald test for logistic regression analysis.
Arg, Arginine; Gln, glutamine; His, histidine; Leu, leucine; Met, methionine.
doi:10.1371/journal.pone.0058380.t004

would be reflected better by serum HCV levels before transplantation than by those after transplantation. It is unclear whether this result is specific to LDLT or holds true for both DDLT and LDLT. The significance of pretransplant viral load in DDLT as a predictor for virological response to post-transplant interferon therapy has not been analyzed in most previous studies [10]. Further analysis in patients who receive DDLT could help clarify the underlying mechanism.

Liver transplantation across the ABO blood-type barrier (ABO-incompatible) is generally contraindicated because of the possibility of graft loss caused by antibody-mediated rejection and is performed under exceptional circumstances as a rescue option in an emergent situation. However, ABO-incompatible LDLT has been performed in Japan to overcome organ shortage problems. Recently, rituximab prophylaxis and local infusion of prostaglandin E1 and steroids were established as therapeutic measures for recipients who underwent ABO-incompatible LDLT, and these treatments improved outcomes [46]. Interestingly, in this study, we found that an ABO-mismatched donor is associated with VR to interferon therapy. The reason for this interesting finding is unclear, but it is possible that either subclinical antibody-mediated rejection or drugs such as rituximab and prostaglandin E1 used in ABO-incompatible recipients may contribute to the higher VR to interferon therapy. There is hope that future studies to clarify the basic mechanism underlying this result will lead to a novel strategy to improve the efficacy of interferon therapy in patients with hepatitis C.

Amino acid substitutions of core region of HCV were not associated with treatment response in our analysis. We do not know the reason for the difference of impact of substitution of core aa 70 and aa 91 on virological response to interferon therapy from a previous report, in which SVR rate were significantly higher in transplant recipients with aa 70 of arginine and aa 91 of leucine of core region of HCV [33]. As sample size of both the previous study and our present study are small, and our present study did not assess the other HCV RNA mutations, including ISDR [32] and interferon/ribavirin resistance-determining region [47] in NS5A, and IL28B polymorphism in recipients and donors, further analysis should be required in larger cohorts.

Another aim of this study was to identify predictive variables for adverse events during interferon therapy, but none of the studied factors proved to be statistically significant predictors of withdrawal from the treatment protocol. As patients withdrew from the treatment for diverse reasons, it would be difficult to predict each adverse event before the initiation of interferon therapy. Therefore, careful follow-up during the treatment procedure is important for early detection of adverse events and to prevent progression to severe complications.

In this study, the final outcomes of the treatment including standard interferon plus ribavirin and peginterferon plus ribavirin were analyzed. Difference of the efficacy between standard interferon and peginterferon might affect the results of our present study. We predicted that patients who had virological response to standard interferon would also show the same response to peginterferon, because it is reported that the efficacy of peginterferon plus ribavirin is higher than that of standard interferon plus ribavirin [44,48]. Accordingly, the patients who achieved SVR by standard interferon were included in the present study. On the other hand, all nonresponders and all patients who relapsed by standard interferon plus ribavirin were retreated with peginterferon plus ribavirin, and we analyzed the final outcomes of the peginterferon plus ribavirin therapy. Therefore, we conclude that the difference of treatment regimen has little influence on our results.

In conclusion, SVR to antiviral therapy in patients with recurrent hepatitis C after LDLT is predictable before transplant by serum HCV-RNA level and HCV genotype. In addition, patients who undergo ABO-incompatible LDLT appear to have a better VR to interferon therapy after liver transplantation. Mechanisms underlying these interesting results are unknown at present, but these findings are likely to be useful for improved clinical assessment of patients with hepatitis C after liver transplantation, and could lead to development of new strategies for better outcomes in LDLT recipients with the HCV genotype 1 and/or a higher pretransplant viral load.

Author Contributions

Conceived and designed the experiments: YU HM. Performed the experiments: YU TK YO KO AY KH YF AMH HH HM. Analyzed the data: YU ST. Contributed reagents/materials/analysis tools: YU TK YO KO AY KH YF AMH HH HM. Wrote the paper: YU HM SU TC.

References

1. Berenguer M, Prieto M, San Juan F, Rayon JM, Martinez F, et al. (2002) Contribution of donor age to the recent decrease in patient survival among HCV-infected liver transplant recipients. Hepatology 36: 202–210.
2. Feray C, Caccamo L, Alexander GJ, Ducot B, Gugenheim J, et al. (1999) European collaborative study on factors influencing outcome after liver transplantation for hepatitis C. European Concerted Action on Viral Hepatitis (EUROHEP) Group. Gastroenterology 117: 619–625.
3. Forman LM, Lewis JD, Berlin JA, Feldman HI, Lucey MR (2002) The association between hepatitis C infection and survival after orthotopic liver transplantation. Gastroenterology 122: 889–896.
4. Gane E (2003) The natural history and outcome of liver transplantation in hepatitis C virus-infected recipients. Liver Transpl 9: S28–34.
5. Prieto M, Berenguer M, Rayon JM, Cordoba J, Arguello L, et al. (1999) High incidence of allograft cirrhosis in hepatitis C virus genotype 1b infection following transplantation: relationship with rejection episodes. Hepatology 29: 250–256.
6. Sanchez-Fueyo A, Restrepo JC, Quinto L, Bruguera M, Grande L, et al. (2002) Impact of the recurrence of hepatitis C virus infection after liver transplantation on the long-term viability of the graft. Transplantation 73: 56–63.
7. Velidedeoglu E, Mange KC, Frank A, Abt P, Desai NM, et al. (2004) Factors differentially correlated with the outcome of liver transplantation in hcv+ and HCV- recipients. Transplantation 77: 1834–1842.
8. Gordon FD, Kwo P, Vargas HE (2009) Treatment of hepatitis C in liver transplant recipients. Liver Transpl 15: 126–135.
9. Terrault NA (2008) Hepatitis C therapy before and after liver transplantation. Liver Transpl 14 Suppl 2: S58–66.
10. Berenguer M (2008) Systematic review of the treatment of established recurrent hepatitis C with pegylated interferon in combination with ribavirin. J Hepatol 49: 274–287.
11. Berardi S, Lodato F, Gramenzi A, D'Errico A, Lenzi M, et al. (2007) High incidence of allograft dysfunction in liver transplanted patients treated with pegylated-interferon alpha-2b and ribavirin for hepatitis C recurrence: possible de novo autoimmune hepatitis? Gut 56: 237–242.
12. Fernandez I, Ulloa E, Colina F, Abradelo M, Jimenez C, et al. (2009) Incidence, risk factors, and outcome of chronic rejection during antiviral therapy for posttransplant recurrent hepatitis C. Liver Transpl 15: 948–955.
13. Stanca CM, Fiel MI, Kontorinis N, Agarwal K, Emre S, et al. (2007) Chronic ductopenic rejection in patients with recurrent hepatitis C virus treated with pegylated interferon alfa-2a and ribavirin. Transplantation 84: 180–186.
14. Berenguer M, Palau A, Fernandez A, Benlloch S, Aguilera V, et al. (2006) Efficacy, predictors of response, and potential risks associated with antiviral therapy in liver transplant recipients with recurrent hepatitis C. Liver Transpl 12: 1067–1076.
15. Carrion JA, Navasa M, Garcia-Retortillo M, Garcia-Pagan JC, Crespo G, et al. (2007) Efficacy of antiviral therapy on hepatitis C recurrence after liver transplantation: a randomized controlled study. Gastroenterology 132: 1746–1756.
16. Neumann U, Puhl G, Bahra M, Berg T, Langrehr JM, et al. (2006) Treatment of patients with recurrent hepatitis C after liver transplantation with peginterferon alfa-2B plus ribavirin. Transplantation 82: 43–47.
17. Oton E, Barcena R, Moreno-Planas JM, Cuervas-Mons V, Moreno-Zamora A, et al. (2006) Hepatitis C recurrence after liver transplantation: Viral and

histologic response to full-dose PEG-interferon and ribavirin. Am J Transplant 6: 2348–2355.
18. Picciotto FP, Tritto G, Lanza AG, Addario L, De Luca M, et al. (2007) Sustained virological response to antiviral therapy reduces mortality in HCV reinfection after liver transplantation. J Hepatol 46: 459–465.
19. Rodriguez-Luna H, Khatib A, Sharma P, De Petris G, Williams JW, et al. (2004) Treatment of recurrent hepatitis C infection after liver transplantation with combination of pegylated interferon alpha2b and ribavirin: an open-label series. Transplantation 77: 190–194.
20. Sharma P, Marrero JA, Fontana RJ, Greenson JK, Conjeevaram H, et al. (2007) Sustained virologic response to therapy of recurrent hepatitis C after liver transplantation is related to early virologic response and dose adherence. Liver Transpl 13: 1100–1108.
21. Bacon BR, Gordon SC, Lawitz E, Marcellin P, Vierling JM, et al. (2011) Boceprevir for previously treated chronic HCV genotype 1 infection. N Engl J Med 364: 1207–1217.
22. Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, et al. (2011) Telaprevir for previously untreated chronic hepatitis C virus infection. N Engl J Med 364: 2405–2416.
23. Poordad F, McCone J, Jr., Bacon BR, Bruno S, Manns MP, et al. (2011) Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med 364: 1195–1206.
24. Zeuzem S, Andreone P, Pol S, Lawitz E, Diago M, et al. (2011) Telaprevir for retreatment of HCV infection. N Engl J Med 364: 2417–2428.
25. Garg V, van Heeswijk R, Lee JE, Alves K, Nadkarni P, et al. (2011) Effect of telaprevir on the pharmacokinetics of cyclosporine and tacrolimus. Hepatology 54: 20–27.
26. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, et al. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461: 399–401.
27. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, et al. (2009) IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41: 1100–1104.
28. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, et al. (2009) Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41: 1105–1109.
29. Charlton MR, Thompson A, Veldt BJ, Watt K, Tillmann H, et al. (2011) Interleukin-28B polymorphisms are associated with histological recurrence and treatment response following liver transplantation in patients with hepatitis C virus infection. Hepatology 53: 317–324.
30. Fukuhara T, Taketomi A, Motomura T, Okano S, Ninomiya A, et al. (2010) Variants in IL28B in liver recipients and donors correlate with response to peg-interferon and ribavirin therapy for recurrent hepatitis C. Gastroenterology 139: 1577–1585, 1585 e1571–1573.
31. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, et al. (2005) Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 48: 372–380.
32. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, et al. (1996) Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334: 77–81.
33. Fukuhara T, Taketomi A, Okano S, Ikegami T, Soejima Y, et al. (2010) Mutations in hepatitis C virus genotype 1b and the sensitivity of interferon-ribavirin therapy after liver transplantation. J Hepatol 52: 672–680.
34. Ueda Y, Takada Y, Haga H, Nabeshima M, Marusawa H, et al. (2008) Limited benefit of biochemical response to combination therapy for patients with recurrent hepatitis C after living-donor liver transplantation. Transplantation 85: 855–862.
35. Ueda Y, Takada Y, Marusawa H, Egawa H, Uemoto S, et al. (2010) Individualized extension of pegylated interferon plus ribavirin therapy for recurrent hepatitis C genotype 1b after living-donor liver transplantation. Transplantation 90: 661–665.
36. Ueda Y, Marusawa H, Kaido T, Ogura Y, Oike F, et al. (2010) Effect of maintenance therapy with low-dose peginterferon for recurrent hepatitis C after living donor liver transplantation. J Viral Hepat.
37. Bedossa P, Poynard T (1996) An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 24: 289–293.
38. Poynard T, Bedossa P, Opolon P (1997) Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet 349: 825–832.
39. Raut V, Mori A, Kaido T, Ogura Y, Taku I, et al. (2012) Splenectomy does not offer immunological benefits in ABO-incompatible liver transplantation with a preoperative rituximab. Transplantation 93: 99–105.
40. Ohno O, Mizokami M, Wu RR, Saleh MG, Ohba K, et al. (1997) New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J Clin Microbiol 35: 201–207.
41. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, et al. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347: 975–982.
42. Ghany MG, Strader DB, Thomas DL, Seeff LB (2009) Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 49: 1335–1374.
43. Hadziyannis SJ, Sette H, Jr., Morgan TR, Balan V, Diago M, et al. (2004) Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med 140: 346–355.
44. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, et al. (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358: 958–965.
45. Thompson AJ, Muir AJ, Sulkowski MS, Ge D, Fellay J, et al. (2010) Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. Gastroenterology 139: 120–129 e118.
46. Egawa H, Teramukai S, Haga H, Tanabe M, Fukushima M, et al. (2008) Present status of ABO-incompatible living donor liver transplantation in Japan. Hepatology 47: 143–152.
47. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, et al. (2008) Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 48: 38–47.
48. Triantos C, Samonakis D, Stigliano R, Thalheimer U, Patch D, et al. (2005) Liver transplantation and hepatitis C virus: systematic review of antiviral therapy. Transplantation 79: 261–268.

Hepatology Research

Hepatology Research 2013; 43: 67–71 doi: 10.1111/j.1872-034X.2012.01020.x

Original Article

Efficacy and safety of prophylaxis with entecavir and hepatitis B immunoglobulin in preventing hepatitis B recurrence after living-donor liver transplantation

Yoshihide Ueda,[1] Hiroyuki Marusawa,[1] Toshimi Kaido,[2] Yasuhiro Ogura,[2] Kohei Ogawa,[2] Atsushi Yoshizawa,[2] Koichiro Hata,[2] Yasuhiro Fujimoto,[2] Norihiro Nishijima,[1] Tsutomu Chiba[1] and Shinji Uemoto[2]

Departments of [1]Gastroenterology and Hepatology and [2]Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Aim: Hepatitis B recurrence after liver transplantation can be reduced to less than 10% by combination therapy with lamivudine (LAM) and hepatitis B immunoglobulin (HBIG). The aim of this study was to evaluate the efficacy and safety of prophylaxis with entecavir (ETV), which has higher efficacy and lower resistance rates than LAM, combined with HBIG in preventing hepatitis B recurrence after living-donor liver transplantation (LDLT).

Methods: Twenty-six patients who received ETV plus HBIG (ETV group) after LDLT for hepatitis B virus (HBV)-related end-stage liver disease were analyzed by comparing with 63 control patients who had received LAM plus HBIG (LAM group).

Results: The survival rates of the patients treated with ETV plus HBIG was 73% after both 1 and 3 years, and there was no statistical difference between the patients in the ETV group and LAM group. No HBV recurrence was detected during the median follow-up period of 25.1 months in the ETV group, whereas the HBV recurrence rate was 4% at 3 years and 6% at 5 years in the LAM group. No patients had adverse effects related to ETV administration.

Conclusion: ETV combined with HBIG provides effective and safe prophylaxis in preventing hepatitis B recurrence after LDLT.

Key words: entecavir, hepatitis B, liver transplantation, living donor

INTRODUCTION

THE RECURRENCE OF hepatitis B virus (HBV) infection after liver transplantation for HBV-related diseases resulted in poor outcomes before the development of effective prophylaxis with lamivudine (LAM) and hepatitis B immunoglobulin (HBIG). Without the prophylaxis, the majority of patients developed recurrent infections due to HBV in the early phases after liver transplantation, and the recurrence resulted in rapidly progressive liver injury, early graft loss and reduced survival.[1–3] The development of prophylaxis dramatically reduced the post-transplant recurrence of hepatitis B and markedly improved prognosis. The most widely used prophylaxis so far has been a combination therapy of LAM and i.v. HBIG.

In the non-transplant setting, the long-term use of LAM resulted in high rates of emergence of resistance to the drug, with rates ranging 14–32% after 1 year and 60–70% after 5 years of treatment. In most cases, the resistance was the result of selection of LAM-resistant mutations in the YMDD motif of the DNA polymerase domain of HBV.[4] Moreover, the emergence of HBV strains with mutations that allow escape from hepatitis B surface antibody (anti-HBs) recognition has been reported in patients vaccinated for HBV,[5,6] in patients with chronic hepatitis B[7,8] and in liver transplant recipients after HBIG administration.[9–11] Therefore, the emergence of LAM resistance and HBIG resistance might

Correspondence: Dr Yoshihide Ueda, Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Email: yueda@kuhp.kyoto-u.ac.jp
Received 13 February 2012; revision 6 March 2012; accepted 28 March 2012.

increase the risk of recurrence during long-term administration of LAM and HBIG, although the rate of HBV recurrence in liver transplant recipients who received prophylaxis with LAM and HBIG for more than 10 years has not been reported to date. At present, several nucleoside analogs are available for the treatment of chronic hepatitis B[4]. Among them, there is entecavir (ETV), a carbocyclic analogue of 2′-deoxyguanosine, which has been shown to have higher efficacy than LAM in patients with chronic hepatitis B. In addition, ETV has a higher genetic barrier to resistance than LAM. The resistance to ETV requires at least three mutations including rtM204V/I, which causes LAM-resistance, rtL180M, and a mutation at one of the following codons: rtT184, rtS202 or rtM250.[4] Therefore, ETV is now used as a first-line therapy in the treatment of chronic hepatitis B worldwide. Data available in the published work suggest that, in transplant recipients, ETV plus HBIG represents a better prophylaxis protocol than LAM plus HBIG for long-term prevention of HBV recurrence after liver transplantation. However, the efficacy and safety of this treatment is largely unknown.

The aim of this study was to evaluate the efficacy and safety of prophylaxis with ETV and HBIG in preventing hepatitis B recurrence after living-donor liver transplantation (LDLT).

METHODS

Patients

WE RETROSPECTIVELY ANALYZED the medical records of 97 patients who underwent LDLT for HBV-related end-stage liver diseases from September 2002 to December 2010. Of these, eight patients were excluded from our study because they had breakthrough hepatitis due to HBV with LAM-resistant mutations and were prescribed LAM plus adefovir before liver transplantation. Accordingly, 89 patients were enrolled in this study.

Prophylaxis with ETV or LAM combined with HBIG

Lamivudine plus HBIG therapy was given to all recipients with HBV-related end-stage liver diseases from September 2002 to November 2006, as reported previously.[12] From December 2006, we changed the protocol for prophylaxis to ETV plus HBIG. ETV at a dose of 0.5 mg/day or LAM at a dose of 100 mg/day was given before transplantation, usually when the patient was referred to the hospital and scheduled for transplantation. Preoperative ETV or LAM prophylaxis was followed by combination with HBIG after transplantation. The first application of HBIG at a dose of 200 IU/kg body mass was administrated i.v. during the anhepatic phase of LDLT, and repeated every day for the first 5 days post-surgery. HBV serological markers were examined at weekly intervals for the first 2 months after the transplant, then at monthly intervals, and 1000 IU of HBIG was periodically administrated to maintain the serum anti-HBs titers at more than 500 IU/L during the first 6 months and 200 IU/L thereafter throughout the follow-up period.[12]

Immunosuppression

Tacrolimus and low-dose steroid therapy were administrated to induce immunosuppression in most patients.[13] Mycophenolate mofetil was administrated to patients who experienced refractory rejection or required reduction of tacrolimus dose due to adverse events. Patients who received ABO blood-type-incompatible transplants were treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone.[14]

Diagnosis of HBV activation

Activation of HBV was diagnosed when hepatitis B surface antigens (HBsAg) and/or HBV DNA became positive in the serum of the patients. After LDLT, HBsAg, anti-HBs and serum HBV DNA were measured at least at 3 monthly intervals. Serological HBV markers, including HBsAg, anti-HBs, hepatitis B core antibody, hepatitis B e antigen (HBeAg) and antibodies to HBeAg (anti-HBe), were measured by chemiluminescent enzyme immunoassay (Fuji Rebio, Tokyo, Japan). Serum HBV DNA titer was analyzed using a commercial polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche, Branchburg, NJ, USA). LAM-resistant YMDD mutant virus was detected by the PCR enzyme-linked mini-sequence assay.[15]

Statistical analysis

Baseline characteristics are shown in Table 1. For continuous variables, medians and ranges are given, and the significance of the data was analyzed with the Wilcoxon rank sum test. For categorical variables, counts are given, and the data were analyzed with the χ^2-test. Survival rates and the rates of patients who showed HBV activation after LDLT were estimated using the Kaplan–Meier method and compared using log–rank tests. $P < 0.05$ was considered significant.

Table 1 Baseline characteristics of 90 patients

	Entecavir + HBIG ($n = 26$)	Lamivudine + HBIG ($n = 63$)	*P*-value
Age (years)	55 (33–68)	53 (26–64)	0.062†
Men/women	19/7	46/17	0.995‡
Primary disease			0.595‡
Acute liver failure	6 (23%)	9 (14%)	
Liver cirrhosis, HCC^-	6 (23%)	20 (32%)	
Liver cirrhosis, HCC^+	14 (54%)	34 (54%)	
HBV markers before LDLT			
$HBsAg^+$	24 (92%)	61 (97%)	0.350‡
$HBeAg^+$	6 (23%)	18 (29%)	0.595‡
HBV DNA before LDLT	<2.6 (<2.6–7.6<)	3.7 (<2.6–7.6<)	0.010†
<2.6 log IU/mL	14 (54%)	19 (30%)	0.024‡
Follow-up period (months)	25.1 (0.2–58.6)	70.6 (0.5–109.2)	<0.001†

Qualitative variables are shown in number; and quantitative variables expressed as median (range).
†Wilcoxon rank sum test.
‡χ^2-Test.
HBeAg, hepatitis B e antigen; HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LDLT, living-donor liver transplantation.

RESULTS

Patient characteristics

TWENTY-SIX PATIENTS who received ETV plus HBIG (ETV group) after LDLT for HBV-related end-stage liver disease were included in this study. Baseline characteristics of these patients are listed in Table 1 and compared with those of 63 control recipients who received LAM plus HBIG (LAM group) at our institute already present in our database. The two groups of patients did not differ significantly by age, sex, primary diseases or serological markers for HBV before LDLT. Serum HBV DNA levels before LDLT were significantly lower in the ETV group than in the LAM group. Fourteen of 26 patients (54%) showed less than 2.6 log IU/mL of serum HBV DNA in the ETV group. Median follow-up period was 25.1 months (range, 0.2–58.6) in the ETV group, whereas it was 70.6 months (range, 0.5–109.2) in the LAM group.

Efficacy and safety of prophylaxis with ETV plus HBIG

Survival rates of the patients treated with ETV plus HBIG estimated by Kaplan–Meier analysis was 73% at both 1 and 3 years (Fig. 1a). There was no difference between the ETV group and the LAM group, in which survival rates were 81% at 1 year, 78% at 3 years and 73% at

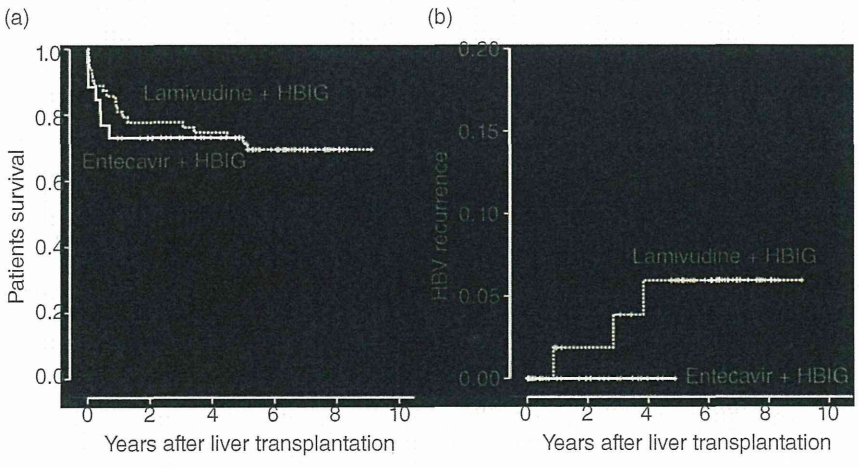

Figure 1 (a) Post-transplantation survival rates and (b) hepatitis B virus (HBV) recurrence after living-donor liver transplantation in HBV positive recipients who received entecavir and hepatitis B immunoglobulin (HBIG) (solid line), or lamivudine and HBIG (dotted line), estimated by Kaplan–Meier method.

5 years. Causes of death in patients in the ETV group were pneumonia ($n = 2$), sepsis ($n = 1$), pulmonary hemorrhage ($n = 1$), cerebral hemorrhage ($n = 1$), graft liver failure ($n = 1$) and multiple organ failure ($n = 1$), none of which were related to ETV. No HBV recurrence was detected in the median follow-up period of 25.1 months in the ETV group, whereas the HBV recurrence rate was 2% at 1 year, 4% at 3 years and 6% at 5 years in the LAM group (Fig. 1b). Three patients in the LAM group had HBV recurrence at 10, 34 and 46 months after LDLT. The emergence of HBV with LAM-resistant mutations in the YMDD motif was confirmed in two of the three patients. HBV mutations of another patient could not be determined because of the low level of serum HBV DNA. As the follow-up period of the ETV group was shorter than that of the LAM group and the HBV recurrence in the LAM group occurred in long-term follow-up after LDLT, the rate of HBV recurrence was not significantly different between the ETV and LAM groups. No patients had adverse events due to ETV administration.

DISCUSSION

IN THIS STUDY, we demonstrated that ETV combined with HBIG provides effective and safe prophylaxis in preventing hepatitis B recurrence after LDLT.

Two studies of patients receiving a combination of ETV and HBIG after liver transplantation have been previously reported.[16,17] One study demonstrated that 30 recipients who received ETV plus HBIG prophylaxis had no recurrence of HBV and no adverse effect relating to ETV.[17] The other study showed that no HBV recurrence was observed in two recipients with HBV-associated cirrhosis receiving ETV, tenofovir and HBIG.[16] Both studies showed the efficacy and safety of prophylaxis with ETV and HBIG in preventing short-term recurrence of HBV after liver transplantation. The current study confirmed their results for longer follow-up periods. Our results showed that prophylaxis with ETV and HBIG has similar efficacy and safety to that with LAM and HBIG, but did not show any further advantage of ETV compared to LAM treatment. Longer follow up might be needed to reveal the difference of HBV recurrence rate. One characteristic of our present report is that all patients in this study underwent LDLT. Our results suggest that prophylaxis with ETV and HBIG in patients after LDLT has similar efficacy and safety to patients after deceased-donor liver transplantation demonstrated in the previous reports.[16,17] More recently, efficacy of ETV monotherapy in preventing recurrence of HBV for liver transplant recipients with chronic hepatitis B was reported.[18] The study demonstrated that most patients showed disappearance of HBsAg and undetectable serum HBV DNA after liver transplantation without HBIG. Although long-term efficacy of ETV monotherapy needs be confirmed, both our data and previous reports suggest that ETV is an effective and safe antiviral agent in the post-transplant setting.

ACKNOWLEDGMENTS

THIS WORK WAS supported by Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (no. 21229009 and 23590972), Health and Labor Sciences Research Grants for Research on Intractable Diseases, and Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan, and a grant from Bristol-Myers-Squibb.

REFERENCES

1 Davies SE, Portmann BC, O'Grady JG *et al.* Hepatic histological findings after transplantation for chronic hepatitis B virus infection, including a unique pattern of fibrosing cholestatic hepatitis. *Hepatology* 1991; **13**: 150–7.
2 O'Grady JG, Smith HM, Davies SE *et al.* Hepatitis B virus reinfection after orthotopic liver transplantation. Serological and clinical implications. *J Hepatol* 1992; **14**: 104–11.
3 Todo S, Demetris AJ, Van Thiel D, Teperman L, Fung JJ, Starzl TE. Orthotopic liver transplantation for patients with hepatitis B virus-related liver disease. *Hepatology* 1991; **13**: 619–26.
4 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
5 Carman WF, Zanetti AR, Karayiannis P *et al.* Vaccine-induced escape mutant of hepatitis B virus. *Lancet* 1990; **336**: 325–9.
6 Hsu HY, Chang MH, Liaw SH, Ni YH, Chen HL. Changes of hepatitis B surface antigen variants in carrier children before and after universal vaccination in Taiwan. *Hepatology* 1999; **30**: 1312–7.
7 Kohno H, Inoue T, Tsuda F, Okamoto H, Akahane Y. Mutations in the envelope gene of hepatitis B virus variants co-occurring with antibody to surface antigen in sera from patients with chronic hepatitis B. *J Gen Virol* 1996; **77** (Pt 8): 1825–31.
8 Yamamoto K, Horikita M, Tsuda F *et al.* Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. *J Virol* 1994; **68**: 2671–6.

9 Carman WF, Trautwein C, van Deursen FJ *et al.* Hepatitis B virus envelope variation after transplantation with and without hepatitis B immune globulin prophylaxis. *Hepatology* 1996; **24**: 489–93.
10 Ghany MG, Ayola B, Villamil FG *et al.* Hepatitis B virus S mutants in liver transplant recipients who were reinfected despite hepatitis B immune globulin prophylaxis. *Hepatology* 1998; **27**: 213–22.
11 Ueda Y, Marusawa H, Egawa H *et al.* De novo activation of HBV with escape mutations from hepatitis B surface antibody after living donor liver transplantation. *Antivir Ther* 2011; **16**: 479–87.
12 Ali H, Egawa H, Uryuhara K *et al.* Prevention of hepatitis B virus recurrence after living donor liver transplantation. *Transplant Proc* 2004; **36**: 2764–7.
13 Ueda Y, Takada Y, Haga H *et al.* Limited benefit of biochemical response to combination therapy for patients with recurrent hepatitis C after living-donor liver transplantation. *Transplantation* 2008; **27** (85): 855–62.
14 Raut V, Mori A, Kaido T *et al.* Splenectomy does not offer immunological benefits in ABO-incompatible liver transplantation with a preoperative rituximab. *Transplantation* 2012; **15** (93): 99–105.
15 Kobayashi S, Shimada K, Suzuki H *et al.* Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). *Hepatol Res* 2000; **17**: 31–42.
16 Jimenez-Perez M, Saez-Gomez AB, Mongil Poce L, Lozano-Rey JM, de la Cruz-Lombardo J, Rodrigo-Lopez JM. Efficacy and safety of entecavir and/or tenofovir for prophylaxis and treatment of hepatitis B recurrence post-liver transplant. *Transplant Proc* 2010; **42**: 3167–8.
17 Xi ZF, Xia Q, Zhang JJ *et al.* The role of entecavir in preventing hepatitis B recurrence after liver transplantation. *J Dig Dis* 2009; **10**: 321–7.
18 Fung J, Cheung C, Chan SC *et al.* Entecavir monotherapy is effective in suppressing hepatitis B virus after liver transplantation. *Gastroenterology* 2011; **141**: 1212–9.

GASTROENTEROLOGY 2013;■:1–12

Splenectomy Prolongs the Effects of Corticosteroids in Mouse Models of Autoimmune Hepatitis

Q15 RYUTARO MARUOKA,[1,2,*] NOBUHIRO AOKI,[1,2,*] MASAHIRO KIDO,[1,2] SATORU IWAMOTO,[1,2] HISAYO NISHIURA,[1,2] AKI IKEDA,[1,2] TSUTOMU CHIBA,[2] and NORIHIKO WATANABE[1,2]

[1]Center for Innovation in Immunoregulative Technology and Therapeutics and [2]Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Q4 Q5 **BACKGROUND & AIMS:** Most patients with autoimmune hepatitis (AIH) initially respond to treatment with corticosteroids but often experience a relapse after treatment is withdrawn. BALB/c mice with disruption of programmed cell death 1 (*PD-1*$^{-/-}$ mice) that undergo thymectomy 3 days after birth develop a deregulated immune system, have reduced numbers of Foxp3^{+} regulatory T cells, and develop fulminant hepatic failure that resembles acute-onset AIH in humans. We examined whether splenectomy overcomes corticosteroid insufficiency and reduces the severity of AIH in these mice. We also developed a mouse model of chronic AIH to investigate the effects of splenectomy. **METHODS:** After thymectomy, BALB/c *PD-1*$^{-/-}$ mice were treated with dexamethasone before or after induction of AIH; splenectomy was performed in mice that had and had not been treated with dexamethasone. Neonatal C57BL/6 *PD-1*$^{-/-}$ mice underwent thymectomy to create a model of chronic AIH. **RESULTS:** Injection of dexamethasone before or after induction of AIH prevented development of fatal AIH in BALB/c *PD-1*$^{-/-}$ mice. However, injection of dexamethasone after induction of AIH did not suppress splenic production of follicular helper T cells, and discontinuation of dexamethasone led to a relapse of AIH. Splenectomy (even without administration of dexamethasone) prevented AIH. Neonatal C57BL/6 *PD-1*$^{-/-}$ mice that underwent thymectomy developed chronic hepatitis with fibrosis and hypergammaglobulinemia and produced antinuclear antibodies; AIH was found to be induced in the spleen. Splenectomy reduced liver inflammation in these mice and in BALB/c *PD-1*$^{-/-}$ mice with AIH. **CONCLUSIONS: AIH can be induced in mice via disruption of PD-1 and thymectomy; these cause the same disruptions in immune regulation in BALB/c and C57BL/6 mice but produce different phenotypes. Splenectomy overcomes corticosteroid insufficiency in mice and prolongs the effects of dexamethasone.**

Keywords: Autoimmunity; ANA; Necrosis; T-Cell Response.

Administration of corticosteroids is the first-line therapy for patients with autoimmune hepatitis (AIH). The majority of patients initially respond well to corticosteroids, alone or in combination with azathioprine.[1–4] After initial remission, maintenance therapy is continued for years. However, long-term treatment is discontinued in 13% of patients with AIH because of drug-related side effects.[5,6] Half of patients with AIH who achieve remission experience a relapse within 6 months after withdrawal of corticosteroids, and multiple relapses are associated with a poor prognosis.[7,8] Even when liver inflammation disappears completely, 13% of those patients eventually experience a relapse.[9] In addition to the difficulty in sustaining remission, a recent study showed that the long-term mortality of patients with AIH due to liver disease is greater than that of the general population.[10] Notably, in Europe and the United States, patients with AIH account for 4% of liver transplants.[4] To find clues to overcoming the therapeutic insufficiency of corticosteroids, preclinical animal models for detailed examination are needed.

AIH is characterized by mononuclear cell infiltration in the liver and elevated levels of gamma globulins as well as by the production of a variety of characteristic autoantibodies, including antinuclear antibodies (ANA).[1–3] Liver-infiltrating T cells are considered the primary disease mediators of inflammatory liver damage, and circulating autoantibodies are diagnostic hallmarks.[1–3] However, clinical manifestations are varied in patients with AIH, ranging from nonsymptomatic mild chronic hepatitis to fulminant hepatic failure.[1–3] It is unclear whether the varied clinical manifestations of AIH result from the same immune dysregulation.

Recently, we developed the first mouse model of spontaneous AIH.[11,12] In *programmed cell death 1*–deficient (*PD-1*$^{-/-}$) mice on the BALB/c background that underwent neonatal thymectomy (NTx) 3 days after birth, immune dysregulation by a concurrent loss of naturally arising Foxp3^{+} regulatory T cells (Tregs) and PD-1–mediated signaling induced fatal AIH resembling acute-onset AIH, presenting in humans as fulminant hepatic failure.[11]

BASIC AND TRANSLATIONAL

*Authors share co-first authorship.

Abbreviations used in this paper: AIH, autoimmune hepatitis; ALT, alanine aminotransferase; ANA, antinuclear antibody; AST, aspartate aminotransferase; DEX, dexamethasone; ELISA, enzyme-linked immunosorbent assay; GC, germinal center; HAI, histological activity index; Ig, immunoglobulin; NTx, neonatal thymectomy; NTx–PD-1$^{-/-}$ mice, PD-1–deficient BALB/c mice that underwent thymectomy 3 days after birth; PBS, phosphate-buffered saline; PD-1, programmed cell death 1; PNA, peanut agglutinin; SD, standard deviation; TCR, T-cell receptor; T_{FH}, follicular helper T; TNF, tumor necrosis factor; Treg, regulatory T cell.

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2013.03.011