Given the high cost and serious side-effects associated with IFN-based treatment, it is clinically important to predict the response to therapy for an individual patient. Predictors can be classified into host factors, such as age, sex, obesity, ethanol consumption, liver fibrosis and a single nucleotide polymorphism (SNP) near the interleukin-28B (*IL-28B*) gene,[9–11] and viral factors, such as viral load, genotypes and polymorphisms in some regions of the HCV genome.

Some regions associated with sensitivity to IFN-based treatment have been identified, including: (i) amino acids (a.a.) 70 and 91 in the core region; (ii) a.a. 2209–2248, known as the IFN sensitivity-determining region (ISDR); and (iii) a.a. 2334–2379, known as the IFN and ribavirin resistance-determining region (IRRDR) in the *NS5A* gene. In brief, Akuta *et al.* reported that a.a. substitutions at positions 70 and 91 in the core region are associated with poor virological response to PEG IFN-α and ribavirin therapy.[12,13] Enomoto *et al.* showed that the sequence variation in the ISDR is associated with good response to IFN-based therapy.[14,15] El-Shamy *et al.* reported that a high degree of sequence heterogeneity in the IRRDR is a useful predictor of a favorable response to PEG IFN-α and ribavirin.[16,17]

Although the relations of variations in the core region, ISDR and IRRDR sequences of the HCV genome to the virological response to IFN-α and ribavirin therapy have been extensively studied, changes in sequences of these regions during treatment remain to be fully evaluated. To the best of our knowledge, no previous study has assessed changes in IRRDR variations. In addition, it remains to be elucidated whether changes in sequences of the three regions caused by IFN-α and ribavirin therapy can affect sensitivity to treatment.

The aims of this study were: (i) to investigate the rates of a.a. mutations in the core region, ISDR and IRRDR during and after IFN-α and ribavirin therapy in patients with chronic HCV genotype 1 infection who did not have SVR; and (ii) to determine whether treatment-related changes in sequences of these three regions can influence therapeutic outcomes when retreatment with IFN-α and ribavirin is subsequently begun.

METHODS

Patients

THE SUBJECTS WERE 25 patients with chronic HCV genotype 1 infection who had not had SVR to treatment with IFN-α and ribavirin, and subsequently received retreatment for a longer duration (Fig. 1). Patients with other hepatitis virus infections, HIV co-infection, autoimmune liver diseases or alcoholic liver injury were excluded from the study. Informed consent was obtained from each patient. Procedures of the study were in accord with the Declaration of Helsinki of 1975 (1983 revision) and were approved by our hospital's ethics committee.

Treatment regimen

For initial combination therapy, 11 patients received (non-pegylated) IFN-α-2b and ribavirin for 24 weeks, and 14 received PEG IFN-α-2b and ribavirin for 48 weeks. For retreatment, 11 patients received PEG IFN-α-2b and ribavirin, and 14 received PEG IFN-α-2a and ribavirin for 48–72 weeks.

IFN-α-2b (Intron-A; MSD, Tokyo, Japan) was given by s.c. injection at a dose of 6 MU every day for the first 2 weeks, followed by three times a week for 22 weeks. PEG IFN-α-2b (Peg-Intron; MSD) was given by s.c. injection at a dose of 1.5 μg/kg once a week for 48–72 weeks. PEG IFN-α-2a (Pegasys; Chugai Pharmaceutical, Tokyo, Japan) was given by s.c. injection at a dose of 180 μg once a week for 48–72 weeks. The duration of retreatment was basically determined in accordance with the guidelines proposed by the Japanese Study Group for the Standardization of Treatment of Viral Hepatitis.[18] Ribavirin (Rebetol [MSD] or Copegus [Chugai Pharmaceutical]) was given p.o. twice a day at a total dose of 600–1000 mg according to bodyweight. The doses of IFN-α and/or ribavirin were sometimes

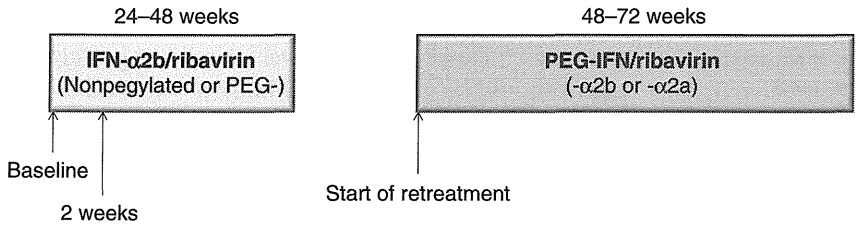

Figure 1 Regimens of treatment and retreatment with IFN-α and ribavirin, and time-points of serum sampling. IFN, interferon; PEG, pegylated.

reduced because of adverse events in accordance with the manufacturers' recommendations. Subjects were excluded if they did not receive 80% or more of the total planned doses of either PEG IFN-α or ribavirin.

An SVR was defined as undetectable HCV RNA in serum at the end of treatment and 24 weeks post-treatment. A relapse was defined as undetectable HCV RNA at the end of treatment and reappearance of HCV RNA within 24 weeks after the end of treatment. Non-response was defined as detectable HCV RNA at the end of treatment.

Routine laboratory examinations

Blood cell counts and biochemical tests were done by standard procedures. The genotype of HCV was determined by serological grouping of serum antibodies on enzyme-linked immunosorbent assay (SRL, Tokyo, Japan), as described previously.[19] HCV RNA was measured by the TaqMan HCV test (Roche Diagnostics, Tokyo, Japan).[20]

Sequence analyses of HCV RNA

As shown in Figure 1, serum samples were taken: (i) at the start of the first treatment (baseline); (ii) at 2 weeks of the first treatment; and (iii) at the start of retreatment and were stored at −80 °C before being tested. HCV RNA was extracted from 100 μL serum by means of a Sepa-Gene RV-R nucleic acid extracting kit (Sanko Junyaku, Tokyo, Japan) and reverse transcribed to complementary DNA by means of SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA) with a random hexamer primer.

The core region of HCV RNA was amplified by nested polymerase chain reaction (PCR) as described previously.[21] The *NS5A* region including ISDR/IRRDR was amplified as described previously.[22] The amplified PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) after agarose gel electrophoresis. The sequences of the amplified fragments were analyzed by direct sequencing using a BigDye Terminator ver. 3.1 Cycle Sequencing Kit and an ABI 3730 xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The a.a. sequences were deduced and aligned using Win software (Genetyx ver. 10; SDC, Tokyo, Japan). All sequence data were compared with the prototype HCV genotype 1b, HCV-J (GenBank accession no. D90208).[23]

In some patients showing changes in the types of core region, ISDR or IRRDR, we performed cloning before sequencing for paired samples before the first and second rounds of treatment. The PCR products were ligated into pGEM-T Easy Vector DH5α (Promega, Madison, WI, USA), and transformed into *Escherichia coli*-competent cells. Transformants were grown overnight on LB/ampicillin/IPTG/X-gal plates, and 15–20 individual clones from each sample were sequenced.

Arginine at position 70 (70R) and leucine at position 91 (91L) in the core region are referred to herein as wild type or sensitive type, and glutamine at position 70 (70Q) and methionine at position 91 (91M) are referred to as mutant type or resistant type. The a.a. sequence variations of the ISDR were classified into resistant type (0–1 mutations) and sensitive type (≥2 mutations). The a.a. sequence variations of the IRRDR were classified into resistant type (<6 mutations) and sensitive type (≥6 mutations).

IL-28B genotyping

We examined SNP of the *IL-28B* gene in patients who consented to genome analysis. Genomic DNA was extracted from whole blood samples obtained from each patient. A genetic polymorphism located upstream of the *IL-28B* gene, rs8099917, was determined by a TaqMan PCR assay.[24] Heterozygosity (T/G) or homozygosity (G/G) for the minor allele (G) was defined as *IL-28B* minor type, whereas homozygosity for the major allele (T/T) was defined as *IL-28B* major type.

Liver histology

After informed consent was obtained, a liver biopsy was performed for each patient within 6 months before the start of therapy. The histopathological findings were assessed by grading inflammatory activity and staging fibrosis according to the METAVIR scoring system.[25]

Statistical analysis

Statistical analysis was performed with SAS ver. 9.2 for Windows (SAS Institute, Cary, NC, USA). Differences in proportions were tested by Fisher's exact test. A two-tailed *P*-value of less than 0.05 was considered to indicate statistical significance.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper have been submitted to the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank nucleotide sequence databases with the accession numbers AB700211 through AB700340.

RESULTS

Patient characteristics

THE BASELINE CHARACTERISTICS of patients are shown in Table 1. Among the 25 patients who had not had SVR to treatment with IFN-α and ribavirin, 11 (44%) achieved SVR to subsequent retreatment, eight (32%) had relapse and six (24%) had non-response.

Changes in core region

Table 2 and Figure 2 show the sequences or types of the core region, ISDR and IRRDR of the HCV genome at baseline, at 2 weeks of the first treatment and at the start of retreatment. In some serum samples taken at 2 weeks of treatment, HCV RNA was not amplified by PCR, probably because of low viral loads.

At baseline, a.a. 70 in the core region was wild (sensitive) type in 18 patients and mutant (resistant) type in seven. At the start of retreatment, a.a. 70 had changed from wild to mutant type in two (patients 15 and 16) and SVR was not achieved by retreatment.

Amino acid 91 in the core region was wild (sensitive) type in 20 patients and mutant (resistant) type in five patients at baseline. At 2 weeks of the first treatment, the a.a. changed from mutant to wild type in one patient (patient 8), but reverted after treatment. At the start of retreatment, no change in a.a. 91 was found as compared with baseline.

Table 1 Baseline characteristics of patients

No. of patients	25
Age (years)	57 ± 8
Sex (female/male)	13/12
Bodyweight (kg)	59.3 ± 9.8
Previous IFN monotherapy (+/−)	10/15
IL-28B SNP rs8099917 (TT/TG/GG/not determined)	12/9/0/4
Laboratory data	
Alanine aminotransferase (IU/L)	69 ± 41
Albumin (g/dL)	4.0 ± 0.3
γ-Glutamyltransferase (IU/L)	38 ± 24
Platelet count ($\times 10^3/mm^3$)	168 ± 54
HCV RNA (log copies/mL)	6.4 ± 0.3
Liver histology	
Grade of necroinflammation (A0/A1/A2/A3)	1/15/5/1
Stage of fibrosis (F1/F2/F3/F4)	12/6/2/2

Values are represented as means ± standard deviation.
HCV, hepatitis C virus; IFN, interferon; SNP, single nucleotide polymorphism.

Changes in ISDR

At baseline, variations in the ISDR were resistant type (0–1 mutations) in 17 patients and sensitive type (≥2 mutations) in eight. After only 2 weeks of the first treatment, a.a. change was found in two patients: in one (patient 8), the substitutions returned to baseline after treatment, and in the other (patient 19) the substitution persisted thereafter. At the start of retreatment, the ISDR sequences had changed from resistant to sensitive type in two (patients 1 and 2) and SVR was achieved by retreatment, and from sensitive to resistant type in three (patients 19–21) and SVR was not achieved by retreatment. During a mean period of 26 months between the first treatment and retreatment, the rate of a.a. mutation in the ISDR was estimated to be 4.7×10^{-3} changes/site per year.

Changes in IRRDR

At baseline, the variations in the IRRDR were resistant type (<6 mutations) in 19 patients and sensitive type (≥6 mutations) in six. At 2 weeks of the first treatment, the type of the IRRDR changed from resistant to sensitive in one patient (patient 17); the number of mutations in the IRRDR changed from five to six, but reverted after treatment. At the start of retreatment, no change in the IRRDR type was found as compared with baseline. During the period between the first treatment and retreatment, the rate of a.a. mutation in the IRRDR was estimated to be 2.9×10^{-3} changes/site per year.

Clonal sequencing analyses

Table 3 shows the results of clonal sequencing in the patients showing changes in types of the core region or ISDR. In patient 15, the sensitive type of a.a. 70 in the core region was the predominant strain at a prevalence of 100% before the first treatment, and the resistant type became the predominant strain at a prevalence of 90% before the start of retreatment. In patient 2, the resistant type in the ISDR was the predominant strain at a prevalence of 90% before the first treatment, and the sensitive type became the predominant strain at a prevalence of 94% before the start of retreatment. In patient 20, the sensitive type in the ISDR was the predominant strain at a prevalence of 65% before the first treatment, and the resistant type became the predominant strain at a prevalence of 95% before the start of retreatment. In patient 21, the sensitive type in the ISDR was the predominant strain at a prevalence of 100% before the first treatment, and the resistant type became the predominant strain at a prevalence of 69% before the start of retreatment.

Table 2 Changes in sequences of the core region, ISDR and IRRDR of the HCV genome and HCV RNA titer

	HCV-J	Core		ISDR	IRRDR	HCV RNA (log copies/mL)	*IL-28B* genotype rs8099917	Outcome
		70	91	a.a. 2209-2248	a.a. 2234-2379			
		R	L	PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN	VLTESTVSSALAELATKTFGSSGSSAVDSGTATAPPDQASDDGDKG			
1	Baseline	-	-	**----------R----------**	-----NM--------K----------SLN-P-G---T-	5.8		SVR
	2 weeks	-	-	**----------R----------**	-----NM--------K----------SLN-P-G---T-	3.8	T/G	
	Restart	-	-	**-------P-CL----------**	-----NM--------K----------I--------SLN-P-G---T-	6.0		
2	Baseline	Q	-	**---R----------**	----------I---------T-P-----A-	6.6		SVR
	2 weeks	Q	-	**---R----------**	----------I---------T-P-----A-	5.3	T/G	
	Restart	Q	-	**---R-----N----------**	----------I---------T-P-----A-	6.1		
3	Baseline	Q	-	---------R-----V----------	I----------E-------M---S-N-P--N--A-	6.4		SVR
	2 weeks	Q	-	---------R-----V----------	I----------E-------M-I-S-N-P--N--A-	4.2	T/G	
	Restart	Q	-	---------R-----V----------	I----------E-------M---S-N-P--N--A-	6.1		
4	Baseline	Q	-	---------R----------D------K-	----------T-	6.4		SVR
	2 weeks	Q	-	---------R----------D------K-	----------T-	5.4	N.D.	
	Restart	Q	-	---------R----------D------K-	----------T-	5.6		
5	Baseline	-	-	---------R----------	----------A------E--G----M--G----P-----V-	6.6		SVR
	2 weeks	-	-	×	×	3.2	T/T	
	Restart	-	-	---------R----------	----------A------E--G----M--G----P-----V-	6.6		
6	Baseline	-	-	---------R----------	----------P--N--T-	7.1		SVR
	2 weeks	-	-	×	×	3.3	T/T	
	Restart	-	-	---------R----------	----------P--N--T-	5.0		
7	Baseline	-	-	----------R-L---------W--K----N------	-----N----------A----------G----E	6.7		SVR
	2 weeks	-	-	×	×	2.0	T/T	
	Restart	-	-	----------R-L---------W--K----N------	-----N----------A----------G----E	6.7		
8	Baseline	-	M	----------	----------E--------E----G-PL----A-	6.7		SVR
	2 weeks	-	-	---------Y-N----------	I----------T-E--------E----G-PL----A-	3.6	T/G	
	Restart	-	M	----------	----------E--------E----G-PL----A-	6.5		
9	Baseline	-	M	----------	----------G-----A----------R-	6.5		SVR
	2 weeks	×	×	×	×	2.9	T/T	
	Restart	-	M	----------	----------G-----A----------R-	6.5		
10	Baseline	-	-	----------	I----------A----------P-----A-	6.1		SVR
	2 weeks	-	-	----------	I----------A----------P-----A-	2.6	T/T	
	Restart	-	-	----------	I----------A----------P-----A-	6.7		
11	Baseline	-	-	----------	----------S--------G---A-	6.3		SVR
	2 weeks	×	×	----------	----------A----------G---A-	3.0	N.D.	
	Restart	-	-	----------	----------A----------G---A-	6.8		
12	Baseline	-	-	----------	-----N---------N----------A----------T-------	6.3		Relapse
	2 weeks	-	-	----------	-----N---------N----------A----------T-------	4.0	T/T	
	Restart	-	-	----------	-----N---------N----------A----------T-------	6.9		
13	Baseline	-	-	----------	----------LT-T--E--T-	6.6		Relapse
	2 weeks	-	-	----------	----------LT-T--E--T-	5.0	T/T	
	Restart	-	-	----------	----------LT-T--E--T-	7.3		
14	Baseline	-	-	---------R----------	----------A------E---A----------PL-S--T-	6.4		Relapse
	2 weeks	-	-	×	×	3.0	N.D.	
	Restart	-	-	---------R----------	----------A------E---A----------PL-S--T-	5.7		
15	Baseline	-	-	----------	----------E---A----------E	6.7		Relapse
	2 weeks	-	-	----------	----------E---A----------E	5.6	T/T	
	Restart	**Q**	-	----------Q----------	----------E---A----------G-E	6.6		
16	Baseline	-	-	---------R-----V----------	----------E----------N-S-----A-	6.5		Relapse
	2 weeks	-	-	---------R-----V----------	----------E----------N-S-----A-	5.1	T/G	
	Restart	**Q**	-	---------R-E---V----------	----------E----------N-S-----A-	6.4		

Table 2 *Continued*

	HCV-J	Core		ISDR	IRRDR	HCV RNA (log copies/mL)	*IL-28B* genotype rs8099917	Outcome
		70 R	91 L	a.a. 2209-2248 PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN	a.a. 2234-2379 VLTESTVSSALAELATKTFGSSGSSAVDSGTATAPPDQASDDGDKG			
17	Baseline	Q	-	--------AR----------	I--D----------A----------P-----A-	6.5		Relapse
	2 weeks	Q	-	--------AR----------	I--D----P----------A----------P-----A-	4.2	T/T	
	Restart	Q	-	--------AR----------	I--D----------A----------P-----A-	6.9		
18	Baseline	-	M	---------R----------	----------A---M----------RE	6.1		Relapse
	2 weeks	-	M	×	×	3.4	T/T	
	Restart	-	M	----------	----------A---M----------R-	6.9		
19	Baseline	-	M	**--------I------P**----------	----------I----------DR	6.8		Relapse
	2 weeks	-	M	**--------I**----------	----------K------NR	5.1	T/T	
	Restart	-	M	**--------I**----------	----------I----------DR	6.5		
20	Baseline	-	-	**---------R-V**----------	I----------A----------G--T-P--EA-T-	6.1		NR
	2 weeks	-	-	**---------R-V**----------	I----------A----------G--T-P--EA-T-	5.3	T/T	
	Restart	-	-	**----------V**----------	I----------A----------G--T-P--EA-T-	6.6		
21	Baseline	Q	M	**----------R----T**----------	----------E----------	5.7		NR
	2 weeks	Q	M	**----------R----T**----------	----------E----------	5.4	T/G	
	Restart	Q	M	**---------------T**----------	----------E----------	5.8		
22	Baseline	Q	-	---------K----------	----------E----------E---	6.9		NR
	2 weeks	Q	-	---------K----------	----------E----------E---	6.5	N.D.	
	Restart	Q	-	---------K----------	----------E----------E---	6.7		
23	Baseline	Q	-	----------R----------	----------T-P--E--T-	6.0		NR
	2 weeks	Q	-	----------R----------	----------T-P--E--T-	6.1	T/G	
	Restart	Q	-	----------R----------	----------T-P--E--T-	6.7		
24	Baseline	-	-	----------	----------T------E	6.5		NR
	2 weeks	-	-	---------------V----------	----------G----------T------A	5.5	T/G	
	Restart	-	-	---------------V----------	----------S------A	6.2		
25	Baseline	-	-	----------	----------E----------T-------	6.2		NR
	2 weeks	-	-	----------	----------E----------T-------	5.7	T/G	
	Restart	-	-	----------	----------E----------T-------	5.6		

× denotes no amplification of polymerase chain reaction products. Boldface type indicates the region in which amino acid sequence changed.
a.a., amino acid; HCV, hepatitis C virus; IFN, interferon; IRRDR, interferon and ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; N.D., not determined; NR, no response; SVR, sustained virological response.

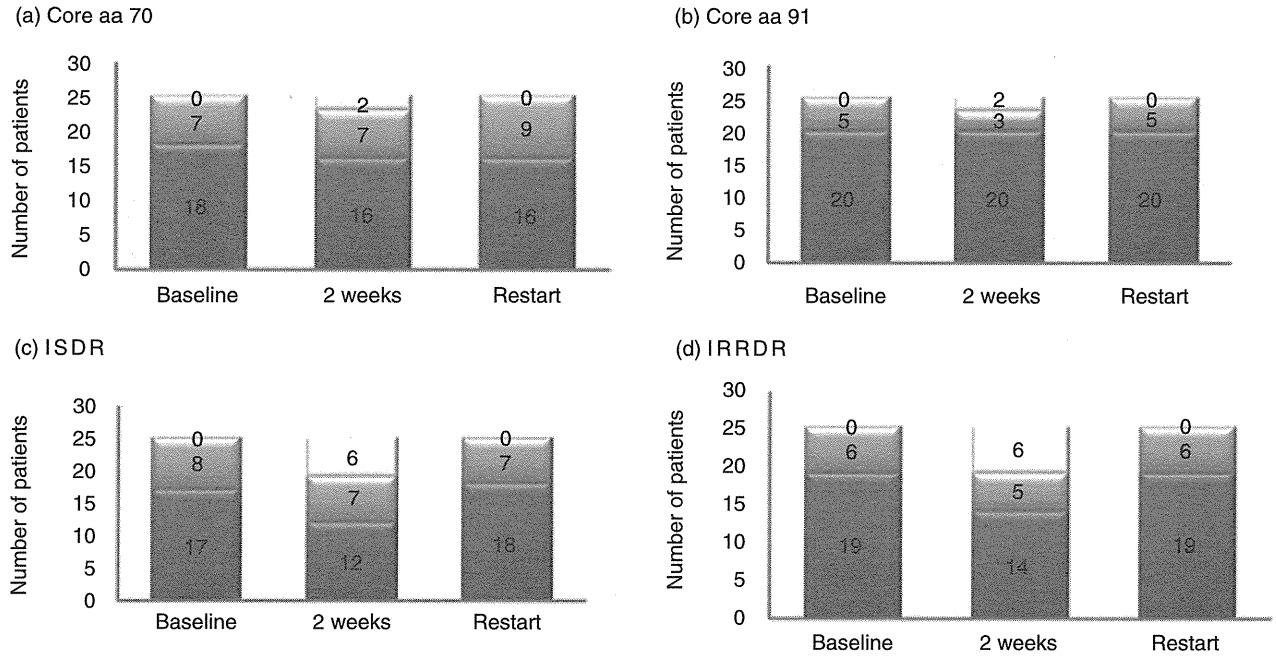

Figure 2 (a) Changes in types of a.a. 70 of the core region (■, wild type; ■, mutant type; □, NA). (b) Changes in types of a.a. 91 of the core region (■, wild type; ■, mutant type; □, NA). (c) Changes in types of ISDR (■, 0–1 mutation; ■, ≥2 mutations; □, NA). (d) Changes in types of IRRDR (■, <6 mutations; ■, ≥6 mutations; □, NA) of the HCV genome at baseline, at 2 weeks of the first treatment, and at the start of retreatment. a.a., amino acid; ISDR, interferon sensitivity-determining region; IRRDR, interferon and ribavirin resistance-determining region; NA, no amplification of polymerase chain reaction products.

Types of core region, ISDR and IRRDR at start of retreatment and treatment outcomes

The proportions of patients with the sensitive type of a.a. 70 and 91 in the core region, ISDR and IRRDR were higher among patients with SVR than among those without SVR (73% vs 57%, 82% vs 79%, 45% vs 14%, and 36% vs 14%, respectively), but the differences did not reach statistical significance, probably because of the small numbers of patients.

DISCUSSION

ONLY A FEW groups have studied changes in sequences of the core region, ISDR and IRRDR of the HCV genome during treatment with IFN-α and ribavirin, although variations in these regions are very important for predicting response to therapy. In this study, the types of a.a. 70 of the core region, ISDR and IRRDR, respectively, changed in two (8%), five (20%) and zero (0%) of the 25 patients with chronic HCV genotype 1 infection who had not had SVR to IFN-α and ribavirin during a mean period of 26 months between the first treatment and subsequent retreatment. Of interest, when the type changed from resistant to sensitive in the core region or ISDR, SVR was achieved by retreatment and vice versa. Our results suggest that sequences of the core region and ISDR of the HCV genome sometimes change during antiviral therapy, and such changes can affect the outcome of retreatment.

However, changes in sequences of the core region and ISDR during antiviral therapy do not solely account for the outcomes of retreatment. For example, in patient 1, in whom the type changed from resistant to sensitive in the ISDR, and SVR was achieved by retreatment, the type of the core region or IRRDR was also sensitive from the beginning. In contrast, it is interesting that patient 2, who had the resistant types of all three regions at baseline, achieved an SVR to retreatment after the type of ISDR changed to sensitive. Another possible explanation is that favorable outcomes can be obtained by a prolonged duration of retreatment, particularly when patients have treatment-sensitive HCV isolates. In fact, the proportions of patients with the sensitive type of core region, ISDR and IRRDR were higher among

Table 3 Clonal sequencing in the patients showing changes in types of the core region or ISDR

	HCV-J	Core				Clones	Prevalence	Sensitive type or resistant type	Outcome
		70 RRQPIPKARR	80 PEGRTWAQPG	90 YPWPLYGNEG	100 LGWAGWLLSP				
15	Baseline	---------Q	----A-----	----------	----------	0/17	0%	Resistant	Relapse
		----------	----A-----	----------	----------	17/17	100%	Sensitive	
	Restart	---------Q	----A-----	----------	----------	18/20	90%	Resistant	
		----------	----A-----	----------	----------	2/20	10%	Sensitive	

	HCV-J	ISDR	Clones	Prevalence	Sensitive type or resistant type	Outcome
		a.a. 2209-2248 PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN				
2	Baseline	----------	1/20	5%	90%: resistant	SVR
		---R------	16/20	80%		
		----------S	1/20	5%		
		---R-------T----------	1/20	5%	10%: sensitive	
		---R----------W----------	1/20	5%		
	Restart	---R----------	1/16	6%	Resistant	
		---R-----N----------	13/16	82%	94%: sensitive	
		---R--Y--N----------	1/16	6%		
		---R--F--N----------	1/16	6%		
20	Baseline	----------	6/20	30%	35%: resistant	NR
		----------V----------	1/20	5%		
		---------R-V----------	12/20	60%	65%: sensitive	
		------Y--R-V----------	1/20	5%		
	Restart	----------V----------	19/20	95%	Resistant	
		----------V--G---T----------	1/20	5%	Sensitive	
21	Baseline	---------T----------	0/20	0%	Resistant	NR
		----------R----T----------	10/20	50%	100%: sensitive	
		----------R-------V----------	8/20	40%		
		----------R----T----------N	1/20	5%		
		----------R----T----------G-	1/20	5%		
	Restart	---------T----------	13/19	69%	Resistant	
		----------S-T----------	4/19	21%	31%: sensitive	
		---------T----------A----	1/19	5%		
		-------A-----S-T----------	1/19	5%		

a.a., amino acid; HCV, hepatitis C virus; IFN, interferon; IRRDR, interferon and ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; N.D., not determined; NR, no response; SVR, sustained virological response.

patients with SVR than among those without SVR, albeit not significantly.

Kurbanov *et al.* reported that in approximately one-fourth of patients with no response to PEG IFN-α and ribavirin, the prevalence of resistant type substitutions at a.a. 70 in the core region increased during treatment, indicating that treatment-induced selection of resistant strain had occurred in such patients.[26] Consistent with the findings of their study, our results of direct sequencing also showed that a.a. 70 in the core region changed from sensitive to resistant type in two patients during and after treatment. To validate the results of direct sequencing, we performed cloning and sequenced 20–25 clones each from eight samples for which the results of direct sequencing and typing by PCR using mutation-specific primers (SRL, Tokyo, Japan)[27] were not identical, namely, a.a. 70 in core region was wild type on direct sequencing and competitive type on PCR typing. The results of cloning analysis, showing that the predominant strain was persistently wild type at a prevalence of 74–100%, were in accord with those of direct sequencing (data not shown). The core substitution at position 70 demonstrates the close association with SNP near the *IL-28B* gene. Miura *et al.* found that a.a. 70 in the core region tended to change from sensitive to resistant type over time in patients with *IL-28B* minor homozygous or heterozygous type.[28] We could not confirm this tendency, because one of the two patients with the time-dependent changes in the core gene had *IL-28B* major type (patient 15), and the other had *IL-28B* minor type (patient 16).

Previous studies reported that the type of ISDR (0–1 or ≥2 mutations) changed in only 5.6–9.1% of patients with chronic HCV genotype 1 infection during the natural course at an average interval of 3.5–13 years.[29–31] Nagayama *et al.* showed that the rates of a.a. mutation during the natural course of disease in the full-length HCV genome and *NS5A* region were $1.23–2.58 \times 10^{-3}$ and $0–3.19 \times 10^{-3}$ changes/site per year, respectively.[32] In the 11 patients for whom serum samples taken 3–6 months after the first treatment were available, we also estimated the rates of a.a. mutation during natural course until the start of retreatment. The estimated rates of a.a. mutation in the ISDR and IRRDR were 2.4×10^{-3} and 0.7×10^{-3} changes/site per year, respectively (data not shown), comparable to the rates reported by Nagayama *et al.*[32] In contrast, during the study period as a whole, which included a period in which patients received antiviral therapy, the rates of a.a. mutation in the ISDR and IRRDR (4.7×10^{-3} and 2.9×10^{-3} changes/site per year, respectively) were much higher than those observed during natural course, suggesting that the mutation rates were increased by the treatment. There is a possibility that the interval length between these two IFN-based therapies in each patient might affect viral sequence change. However, a univariate comparison of patients with sequence change versus patients without sequence change showed that the mean interval between treatments did not differ between the two groups (26 vs 16 months; $P = 0.12$, data not shown).

El-Shamy *et al.* originally reported that less than six a.a. mutations in the IRRDR were significantly associated with a poor response to PEG IFN-α and ribavirin therapy.[16,17] More recently, they updated the cut-off value of the resistant type to less than four mutations.[33] When we reviewed our data according to this new classification, the variations in the IRRDR at baseline were resistant type (<4 mutations) in 10 patients and sensitive type (≥4 mutations) in 15. By the time of starting retreatment, the type of the IRRDR changed in two patients: in one (patient 15) the type of the IRRDR changed from resistant to sensitive, but SVR was not achieved by retreatment, and in the other (patient 18) the type of the IRRDR changed from sensitive to resistant, and SVR was not achieved by retreatment. Further studies are required to determine the relation of changes in the type of IRRDR to the outcomes of PEG IFN-α and ribavirin therapy.

Ribavirin is a synthetic guanosine nucleoside analog that inhibits the replication of various RNA and DNA viruses. Although the exact mechanism of action of ribavirin remains unknown, it may act as an RNA viral mutagen *in vitro*, thereby leading to lethal mutagenesis and error catastrophe.[34,35] Previous *in vivo* studies reported that ribavirin monotherapy brought about a mutagenic effect early during treatment.[36–39] We also observed changes in sequences of the core and/or *NS5A* regions in three patients after only 2 weeks of treatment: in two (patients 8 and 17), the a.a. change was found in the core and/or *NS5A* regions but reverted after treatment, and in the other (patient 19), the change was found in the ISDR and persisted thereafter. However, because our treatment regimen was a combination of IFN-α and ribavirin, a considerable proportion of circulating HCV isolates might be cleared immediately by the antiviral action of IFN-α. Another possibility is that the a.a. changes we observed were not induced by de novo mutations, but by clonal selection of a minor strain, because HCV exists *in vivo* as a heterogeneous but closely related viral population termed a "quasispecies". In particular, changes in quasispecies tend to occur more frequently during treatment. In fact, clonal sequencing

analyses showed that the prevalence of minor strains was 10–35% before the first treatment in patients 2 and 20; moreover, the minor strains before the first treatment became the predominant ones before the start of retreatment.

Our study had an important limitation. To study the mechanism of resistance to IFN-α and ribavirin therapy and the outcomes of retreatment, we included only patients without SVR to treatment. In patients with SVR, the rate of a.a. changes induced by the mutagenic effect of ribavirin might be higher, because Asahina *et al.* showed that the rate of mutations caused by ribavirin correlated with the virological response to IFN-α and ribavirin therapy.[36] However, it is impossible to investigate changes in a.a. in such patients, particularly those who have a rapid virological response. HCV variants containing ribavirin-induced error mutations at a functionally important site may lose their viral fitness or may immediately be eliminated by concurrently administrated IFN-α, leading to underestimation of the true mutagenic potential of ribavirin *in vivo*.

In conclusion, our results suggested that sequences of the core region and ISDR of the HCV genome sometimes change during treatment with IFN-α and ribavirin, and that such changes can affect the outcomes of retreatment.

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Significant background rates of HBV and HCV infections in patients and risks of blood transfusion from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres in Japan: a prospective, individual NAT study of transfusion-transmitted HBV, HCV and HIV infections

Y. Tani,[1] H. Aso,[1] H. Matsukura,[1] K. Tadokoro,[2] A. Tamori,[3] S. Nishiguchi,[4] H. Yoshizawa[5], H. Shibata,[1] & JRC NAT Screening Research Group

[1]*Japanese Red Cross Osaka Blood Center, Osaka, Japan*
[2]*Japanese Red Cross Central Blood Institute, Tokyo, Japan*
[3]*Osaka City University Hospital, Osaka, Japan*
[4]*Hyogo College of Medicine, Hyogo, Japan*
[5]*Hiroshima University, Graduate School of Biomedical Sciences, Hiroshima, Japan*

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Background The Japanese Red Cross (JRC) conducted a prospective study to evaluate the frequency of transfusion-transmitted HBV, HCV and HIV infections to assess the risk of transfusion of blood components routinely supplied to hospitals.

Study Design and Methods Post-transfusion specimens from patients at eight medical institutes were examined for evidence of infection with HBV (2139 cases), HCV (2091) and HIV (2040) using individual nucleic acid amplification testing (NAT). If these specimens were reactive, pre-transfusion specimens were also examined for the virus concerned by individual NAT. In the event that the pre-transfusion specimen was non-reactive, then all repository specimens from implicated donors were tested for the viruses by individual donation NAT. In addition, a further study was carried out to evaluate the risk of transfusion of components from donors with low anti-HBc titres or high anti-HBc with high anti-HBs titres.

Results Transfusion-transmitted HCV and HIV infections were not observed. One case of post-transfusion HBV infection was identified (rate, 0·0004675; 95% CI for the risk of transmission, 1 in 451–41 841). The background rates of HBV, HCV and HIV infections in patients prior to transfusion were 3·4% (72/2139), 7·2% (150/2091) and 0% (0/2040), respectively. Sixty-four anti-HBc- and/or anti-HBs-reactive blood components were transfused to 52 patients non-reactive for anti-HBc or anti-HBs before and after transfusion (rate, 0; 95% CI for the risk of transmission, <1 in 22).

Conclusion This study demonstrated that the current criteria employed by JRC have a low risk, but the background rates of HBV and HCV infections in Japanese patients are significant.

Key words: nucleic acid amplification testing, occult HBV infection, transfusion-transmitted viral infection.

Correspondence: Yoshihiko Tani, MD, PhD, Japanese Red Cross Osaka Blood Center, 2-4-43 Morinomiya, Joto-Ku, Osaka 536-8505, Japan
E-mail: tani@osaka.bc.jrc.or.jp

Introduction

In Japan, all blood components are collected from non-remunerated voluntary donors by the Japanese Red Cross (JRC). Subsequently, the components are screened by serological testing for syphilis, HBV, HCV, HIV, HTLV-1 and human parvovirus B19, and serologically non-reactive components (the criteria to determine the suitability of blood for supply, which are based on infectious markers, are shown in Table 1) are then subjected to nucleic acid amplification testing (NAT) for HBV DNA, HCV RNA, and HIV-1 RNA in a mini-pool (MP) using an automated multiplex assay system (AMPLINAT MPX; Roche Diagnostics) that can amplify these three viruses simultaneously [1]. When all the above-mentioned tests are non-reactive, the blood components are supplied to hospitals. An important point is that unlike some other countries [2], blood components from donors non-reactive for HBsAg and HBV DNA but reactive for anti-HBc titres of <1:32 or anti-HBc titres of ≥1:32 with anti-HBs ≥200 mIU/ml are considered suitable for transfusion in Japan on the basis of a study of the correlation between anti-HBc titres and HBV DNA levels in blood units without detectable HBsAg [3, 4].

Since the introduction of the automated NAT multiplex assay system by the JRC in October 1999, the risk of transmission of HBV, HIV and HCV via transfusion has reduced significantly in Japan [5, 6]. However, several cases of transfusion-transmitted viral infections, especially of HBV, continue to occur each year [7]. This is partly because the doubling time of HBV is longer than that of HCV or HIV [8, 9], and thus, the NAT window period is also longer. The other reason is that occult HBV-infected donors [3] with low anti-HBc titres and low levels of HBV DNA that are not detected by MP-NAT may not be identified [10].

In this study, we randomly selected five JRC blood centres (Hokkaido, Iwate, Osaka, Ehime, and Fukuoka) and eight hospitals within the jurisdiction of these centres and prospectively investigated the risk involved in routine blood transfusion to patients in these hospitals. In addition, we also examined the safety of blood transfusion from anti-HBc-positive donors with anti-HBc titres of <1:32 or anti-HBc titres of ≥1:32 with anti-HBs ≥200 mIU/ml.

Materials and methods

Serological tests on donated blood

All donated blood samples were serologically screened as shown in Table 1.

NAT

The NAT screening system used in Japan has been reported previously by Mine *et al.* [12]. In brief, NAT screening is performed using a multiplex system capable of simultaneous detection of HBV DNA, HCV RNA and HIV-1 RNA to reduce the cost and ensure that the test is completed within 72 h. Samples are tested in MPs of 50 with the ability to detect 185–550 IU/ml for HBV, 3050–5600 IU/ml for HCV

Table 1 Criteria for infectious and other markers

	Serological tests		
Pathogens	**Contents**	**Methods**	**Criteria**
Syphilis	Serodiagnosis	Treponema pallidum particle agglutination (TPPA)[a]	Non-reactive
HIV	Anti-HIV-1/2	Agglutination of gelatin particles coated with recombinant HIV-1/2 proteins[a]	Non-reactive
HCV	Anti-HCV	Passive hemagglutination (PHA[b]) or particle agglutination (PA[a])	Non-reactive
HBV	HBsAg	Reverse passive hemagglutination (RPHA[c])	Non-reactive
	Anti-HBs	PHA[c]	–[d]
	Anti-HBc	Haemagglutination inhibition (HI[c])	
HTLV-1	Anti-HTLV-1	PA[a]	Non-reactive
B19	Anti-B19	Receptor-mediated hemagglutination (RHA[e])	
Others	Serum ALT	Method of Wroblewski and LaDue (11)	≤60 IU/ml

[a]Fujirebio Inc., Tokyo, Japan.

[b]Dainabot Co. Ltd., Tokyo, Japan.

[c]Reagents prepared by JRC.

[d]Blood units with the following profile were excluded from being transfused: 1. Specimen reactive for HBsAg on RPHA, with the result subsequently confirmed by enzyme immune assay (EIA). 2. Specimen reactive for anti-HBc at a dilution of 1:32 or higher on HI and in which anti-HBs is either absent or at a level of not more than 200 mIU/ml.

[e]RHA using reagents prepared by JRC.

and 1650–3300 IU/ml for HIV in donations contained within the pool.

In this study, HBV DNA, HCV RNA and HIV-1 RNA from patients were individually tested using the modified methods of Iizuka *et al.* [4], Okamoto *et al.* [13] and Matsumoto *et al.* [14], respectively, at the JRC NAT centres in Hokkaido and Kyoto. The analytical sensitivity cut-off of ID-NAT was 3·7–11 IU/ml for HBV, 61–112 IU/ml for HCV and 33–66 IU/ml for HIV [1].

Criteria for blood transfusion

The serological test criteria for the release of blood donations in Japan are shown in Table 1. Donations must also be non-reactive for HBV DNA, HCV RNA and HIV RNA on 50-MP-NAT.

Study design

Informed consent was obtained from each patient before transfusion between November 2003 and December 2006 at eight hospitals [Asahikawa Medical College Hospital (Hokkaido); Iwate Medical University Hospital (Iwate); Osaka City University Hospital, Osaka City General Hospital, Osaka Red Cross Hospital (Osaka); National Hospital Organization Shikoku Cancer Center, Ehime Red Cross Hospital (Ehime); and Fukuoka University Hospital (Fukuoka)]. In total, 2139 patients who survived 3 months after transfusion (approximately 40% of patients died of their original disease or complications within 3 months) were enrolled in this study. Their pre-transfusion blood specimens had been collected and cryopreserved in these hospitals (Fig. 1).

Approximately 3 months after blood transfusion, post-transfusion specimens were collected from the patients and individually tested for HBV DNA, HCV RNA and HIV-1 RNA at the JRC NAT centres. In the case of neonates and elderly patients, when the specimen volume was insufficient to perform NAT for all the three viruses, the priority of examination was HBV DNA >HCV RNA >HIV-1 RNA.

If the post-transfusion specimen was non-reactive for all the three viruses, the study was terminated for the patient concerned. However, if the specimen was reactive, the patient's cryopreserved pre-transfusion specimen was tested for the virus concerned by NAT. If the pre-transfusion specimen was reactive, it was concluded that the patient was infected before transfusion. However, if the pre-transfusion specimen was non-reactive, all repository specimens from the implicated donors, which were drawn at the time of blood donation and cryopreserved at the JRC NAT centres, were also tested for the virus concerned by ID-NAT, as reported by Satake *et al.* [15]. If these specimens were non-reactive and the case was restricted to HBV, the remaining pre-transfusion specimen of the patient was serologically tested for anti-HBc, anti-HBs and/or HBsAg using an enzyme immunoassay

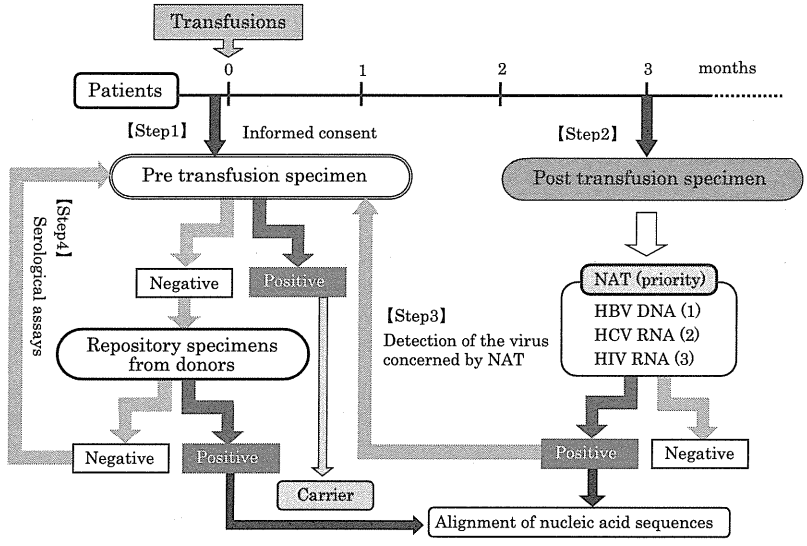

Fig. 1 Study design. Step 1: After obtaining informed consent from patients, pre-transfusion specimens were collected and cryopreserved. Step 2: Approximately 3 months after blood transfusion, post-transfusion specimens were collected from the patients at the eight hospitals and individually tested for HBV DNA, HCV RNA and HIV-1 RNA at the JRC nucleic acid amplification testing (NAT) centres. Step 3: If the post-transfusion specimen was reactive, the patient's pre-transfusion specimen was tested for the virus concerned by NAT. Step 4: If the pre-transfusion specimen was non-reactive (this was restricted to HBV alone), all repository specimens from the donors concerned were also tested for HBV DNA by ID-NAT. If these specimens were non-reactive, the remaining pre-transfusion specimen of the patient was then serologically tested for anti-HBc, anti-HBs and/or HBsAg.

(EIA) system in addition to the methods shown in Table 1.

Assessing the risk of blood transfusion from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres

We randomly selected 247 patients at the Osaka City University Hospital whose post-transfusion specimens were non-reactive for all the three viruses on NAT and 1009 blood components were transfused to these patients. Their pre- and post-transfusion specimens were tested for anti-HBs and anti-HBc. The repository specimens from the implicated donors were also examined to determine anti-HBs and anti-HBc titres and HBV DNA levels.

Results

The risk of transfusion-transmitted HBV, HCV and HIV infections

We examined 2139, 2091 and 2040 post-transfusion specimens for HBV DNA, HCV RNA and HIV-1 RNA, respectively, by NAT. The 2040 post-transfusion specimens were non-reactive for HIV-1 RNA (Table 2). Of the 2091 post-transfusion specimens, 150 specimens (7·2%) were reactive for HCV RNA (Table 2). However, the pre-transfusion specimens from the same 150 patients were also reactive for HCV RNA, indicating that the patients were already infected with HCV prior to the transfusion. Of the 2139 post-transfusion specimens, 73 (3·4%) specimens were reactive for HBV DNA (Table 2). Among these 73 patients, pre-transfusion specimens from 56 patients were reactive for HBV DNA, indicating that these patients were already infected with HBV prior to the transfusion. Pre-transfusion specimens from the remaining 17 patients were non-reactive for HBV DNA. Among these 17 patients, one patient who received 115 units of blood was judged to have transfusion-transmitted HBV infection on the basis of a donor-triggered look-back investigation on a donor, who was found to be reactive for HBV DNA at his next donation. The HBV DNA sequence of this donor was consistent with that of the patient. The repository specimens from the remaining 114 donors were non-reactive for HBV DNA.

Fourteen of the sixteen remaining patients were considered to have late-stage HBV infection because their pre-transfusion specimens were reactive for anti-HBc, and none of the repository specimens from the donors were reactive for HBV DNA. The other two patients were also considered to have late-stage infection because their HBsAg levels were relatively low (Table 3). According to additional information obtained from the hospital, one patient (No. 16 in Table 3) became infected with HBV several years ago and then periodically visited the hospital, and hospital records identified him as being HBsAg positive (AxSYM; Abbott Japan Co., Ltd, Tokyo, Japan). Considering their ages, diseases, therapies [16] administered to the patients, and follow-up observations by the hospitals, these 16 patients were strongly suggested to have occult hepatitis B infection (OBI).

The risk of transfusion of blood components from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres

None of the 1009 repository specimens were reactive for HBV DNA, but 86 of these specimens were reactive in the anti-HBc test at a titre of <1:32 (75 specimens) or ≥1:32

Table 2 Patient ages and HIV-1 RNA, HCV RNA and HBV DNA results

	Age		≤9	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89	≥90	Total
HIV-1 RNA	Post-transfusion	Non-reactive	49	38	56	125	137	345	548	577	157	8	2040
		Reactive	0	0	0	0	0	0	0	0	0	0	0
	Total number		49	38	56	125	137	345	548	577	157	8	2040
HCV RNA	Post-transfusion	Non-reactive	55	38	59	128	134	330	517	518	154	8	1941
		Reactive	0	0	0	1	6	26	40	69	8	0	150
	Pre-transfusion	Non-reactive	0	0	0	0	0	0	0	0	0	0	0
		Reactive	0	0	0	1	6	26	40	69	8	0	150
	Total number		55	38	59	129	140	356	557	587	162	8	2091
HBV DNA	Post-transfusion	Non-reactive	79	43	61	129	135	334	546	574	156	9	2066
		Reactive	0	0	0	1	5	24	18	19	6	0	73
	Pre-transfusion	Non-reactive	0	0	0	0	0	5	4	5	3	0	17
		Reactive	0	0	0	1	5	19	14	14	3	0	56
	Total number		79	43	61	130	140	358	564	593	162	9	2139

Table 3 The details of 16 patients considered to have late-stage HBV infection

				Pre-transfusion				Post-transfusion			
No.	Age	Disease	Therapy	HBsAg	Anti-HBs (mIU/ml)	Anti-HBc	HBV DNA	HBsAg	Anti-HBs	Anti-HBc	HBV DNA
1	64	Heart disease	Operation	+	−	+	−	+	−	NT	+
2	54	Haematologic malignancy	HSCT	−	−	+	−	−	−	+	+
3	77	Gastric cancer	Chemotherapy	−	−	+	−	−	−	NT	+
4	60	AML	Chemotherapy	−	−	+	−	−	−	NT	+
5	56	Haematologic malignancy	HSCT	−	+	+	−	NT	−	+	+
6	76	Macroglobulinemia	HSCT	−	+ 2100	+	−	−	+	+	+
7	72	Oesophageal cancer	Chemotherapy	−	+ 134·2	+	−	−	+	+	+
8	57	Aplastic anaemia	HSCT	−	+ 5·2	+	−	−	+	+	+
9	89	Orthopaedic disorder	Operation	−	+ 34·9	+	−	−	+	+	+
10	70	Heart disease	Operation	−	+ 42·1	+	−	−	+	NT	+
11	77	Intracerebral haemorrhage	Operation	−	+ 1·4	+	−	−	+	NT	+
12	58	Gastric cancer	Chemotherapy	NT	+ 7·5	+	−	−	+	NT	+
13	58	Haematologic malignancy	HSCT	−	+	+	−	NT	NT	NT	+
14	82	Heart disease	Operation	NT	NT	+	−	−	+	+	+
15	80	Cancer	Chemotherapy	+2·52	−	−48·8	−	−1·69	−	+56·8	+
16	67	Gynaecological cancer	Chemotherapy	−1·87	−	−	−	+2·35	−	−	+

+, reactive or positive; −, non-reactive or negative; NT, not tested; HSCT, haematopoietic stem cell transplantation; AML, acute myelocytic leukaemia; HBsAg, anti-HBs and anti-HBc (Nos. 15 and 16) measured by EIA (AxSYM) in the hospital (normal range = HBsAg, S/N of <2·00; anti-HBc, % INH (inhibition) of <50·0) because the specimen volume was not sufficient to perform RPHA, PHA and HI.

Table 4 Analysis of blood components (n = 1009) transfused to 247 randomly selected patients negative for all three viruses on NAT

		Anti-HBc (HI) 2^n									
		0	1	2	3	4	5	6	7	8	Total
Anti-HBs (PHA) 2^n	0	896	14	13	6	3	Excluded from blood transfusion				932
	1	5	2		1	1					9
	2	8		2	3	1					14
	3	3	1	1	2	1					8
	4	3		3		3					9
	5			2	1		1	1		1	6
	6	3		1	1		1	1	1		8
	7	2		2	4	3				1	12
	8	1			1	2					4
	9	1				1			2	1	5
	10	1									1
	11							1			1
	Total	923	17	24	19	15	2	3	3	3	1009
			75				11				

NAT, nucleic acid amplification testing.

Values indicate the number of blood components with titres (2^n) of anti-HBc and anti-HBs transfused.

For example, '14' blood components with titres of anti-HBc and anti-HBs of 2^1 and 2^0, respectively, were transfused to patients.

with an anti-HBs titre of >1:32 that corresponds to 200 mIU/ml (11 specimens) (Table 4). All of the 86 donations met criteria for release for transfusion in Japan (Table 1). Of the 247 patients tested, neither pre- nor post-transfusion specimens from 165 patients were reactive for anti-HBs or anti-HBc, although 52 of these patients received blood components (total of 64) that were serologically reactive for anti-HBs and/or anti-HBc (Fig. 2). In

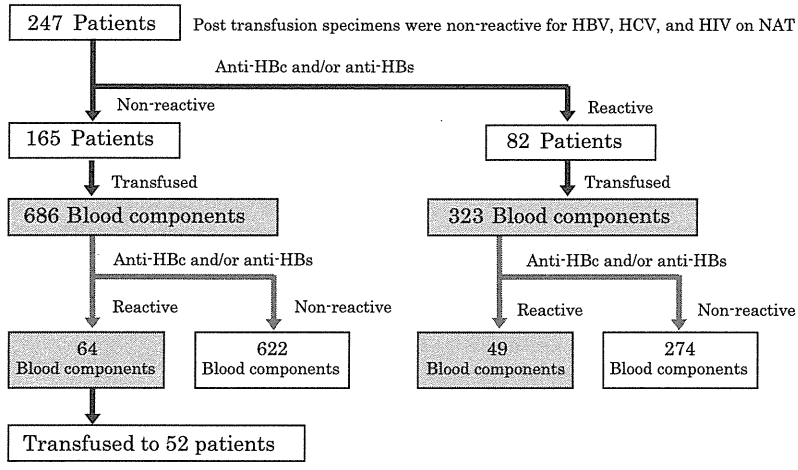

Fig. 2 The risk of transfusion of blood components with low anti-HBc and/or high anti-HBs titres. A total of 247 patients whose post-transfusion specimens were non-reactive for HBV, HCV and HIV on nucleic acid amplification testing were randomly selected, and their pre- and post-transfusion specimens were tested for anti-HBc and anti-HBs. One hundred and sixty-five patients showed non-reactivity for anti-HBc and anti-HBs in both their pre- and post-transfusion specimens, and they were transfused 686 blood components. The remaining 82 patients were reactive for anti-HBc or anti-HBs, and they were transfused 323 blood components. The repository specimens from the donors concerned were examined to determine the anti-HBc and anti-HBs titres. Of the 686 blood components, 64 were reactive for anti-HBc and/or anti-HBs and were transfused to 52 patients whose pre- and post-transfusion specimens were non-reactive for HBV DNA, anti-HBc and anti-HBs.

other words, 64 blood components that were serologically reactive for anti-HBs and/or anti-HBc were transfused to 52 patients, but no reactivity was observed for any of the HBV markers (HBsAg, anti-HBs, anti-HBc and HBV DNA) before and after transfusion.

Discussion

We commenced this study in November 2003 when the tests for post-transfusion hepatitis were not routinely performed in hospitals in Japan, including most of the hospitals that participated in this study. Medical insurance covered the cost of the tests for post-transfusion hepatitis only when a doctor suspected its possibility, and thus, a number of transmissions might have been missed. Similarly, anti-HBc and anti-HBs tests were also not performed before transfusions. Therefore, the JRC conducted this study to try to identify more accurately the transmission rate of infections in all patients receiving blood transfusions in the specified hospitals. The number of patients participating in this study was, however, insufficient to develop statistically significant rates.

Immediately after this study started, a post-transfusion specimen from a patient was found to be reactive for HBV DNA. However, neither the pre-transfusion specimen from the patient nor the repository specimen from the donor concerned was reactive for HBV DNA. Consequently, the remaining pre-transfusion specimen was tested and found to be reactive for anti-HBc. Therefore, it was concluded that the patient had been infected with HBV before transfusion–i.e., he had so-called OBI [17–20]. This demonstrates that OBI is an important issue among patients in Japan, especially in older patients [15] and patients receiving immunosuppressive therapies such as chemotherapy [21–23]. Since the completion of this study, medical insurance has been available to cover the cost of laboratory tests performed to evaluate viral markers of HBV, HCV and HIV (including anti-HBc) in all patients receiving blood transfusions. Furthermore, considering the significant background rates of HCV (7·2%) and HBV (3·4%) infections seen in Japanese patients, the Ministry of Health, Labor and Welfare has developed guidelines for the timing and testing required to support early detection of transfusion-transmitted HBV, HCV and HIV infections (Table 5). Pre-transfusion specimens can be tested for HBsAg, anti-HBs, anti-HBc, HCV core antigen, anti-HCV and anti-HIV. If these specimens are non-reactive for all the viral markers, post-transfusion specimens are tested for HBV DNA, HCV core antigen and anti-HIV. If any of the viral markers are reactive in pre-transfusion specimens, there is no requirement to undertake further testing for the viruses concerned in post-transfusion specimens. Of course, we can cryopreserve pre-transfusion specimens as performed in this study, and if the post-transfusion specimens are reactive for HBV DNA, HCV core antigen or anti-HIV, the cryopreserved pre-transfusion specimens can then be tested for the relevant viral markers.

The reasons for the high background rates of HBV and HCV infections, especially among older patients, are unclear; however, these rates might partly be the result of the reuse of needles and syringes for vaccination during

Table 5 The guideline of test markers for early detection of transfusion associated HBV, HCV and HIV infections in Japan

	Pre-transfusion	Post-transfusion	
Virus	**Test markers**	**When to test**	**Test markers**
HBV	HBsAg Anti-HBs Anti-HBc	Approximately 3 months later	HBV DNA
HCV	HCV core antigen Anti-HCV	1–3 months later	HCV core antigen
HIV	Anti-HIV	2–3 months later	Anti-HIV

childhood to save costs, a practice that lasted until the 1980s, or to the use of plasma anticoagulant products such as fibrinogen and factor VIII, which were not pathogen inactivated in the 1980s and 1990s. In fact, according to a report by Tanaka *et al.* [24], the prevalence of HBV and HCV in first-time blood donors was 0·63% (1·5% estimated for donors above 50 years) and 0·49% (2% estimated for donors above 50 years and 3% for donors above 60 years), respectively.

Transfusion-transmitted HCV or HIV infection was not observed in this study. The patient with confirmed transfusion-transmitted HBV infection was a 61-year-old man with acute myeloid leukaemia, and he underwent hematopoietic stem cell transplantation. On 29 November 2003, the patient received a platelet transfusion. The platelet component was derived from a donor on November 27. The donor's next donation was on 30 December 30 2003, and his blood sample was found to be reactive for HBV DNA using the 50 donation MP-NAT. A look-back study of the donor revealed that HBV DNA was detectable by ID-NAT in the repository specimen collected on 27 November 2003. Serum drawn from the patient on 26 January 2004 (on the same day when the result of look-back was obtained) was also reactive for HBV DNA, but the DNA level was too low (30 copies/ml) to sequence. His pre-transfusion specimen was non-reactive for HBsAg, anti-HBc, anti-HBs and HBV DNA. A total of 115 units of blood including this platelet component transfused to him were implicated, and the repository specimens from the donations were tested for HBV by ID-NAT. All specimens except the one identified previously were non-reactive for HBV DNA. Approximately 4 months later (1 June 2004), the patient's HBV DNA level was elevated (≥1000 copies/ml) along with leukaemia recurrence, and the specified HBV DNA sequences were consistent with those of the original donor. As the HBV DNA level in the patient was monitored, immediate administration of lamivudine (when the HBV DNA level was >1000 copies/ml), a nucleoside analogue reverse transcriptase inhibitor, prevented the development of acute hepatitis.

Despite the implementation of NAT screening, several cases of transfusion-transmitted HBV infection continue to occur each year in Japan [25]. One reason may be that only a few patients are immunized with a hepatitis B vaccine because only selective vaccination against HBV is carried out in Japan (medical staff, coworkers and babies born to HBV carrier mothers). In addition, donors in the early and late stages of HBV infection may have low HBV DNA levels that are detectable in ID-NAT but not by 50-NAT [26]. The patient discussed earlier is a typical case of transfusion of a blood component from a donor with an early acute HBV infection. The risk of HBV transmission identified in this study was 0·0004675 (95% CI for the risk of transmission, 1 in 451–41 841). However, data from donor-triggered look-back studies involving more than 10 000 cases between 2000 and 2004 [15] have been used to assess the residual risks of transfusion-transmission of these three viruses. On the basis of data reported by transfusion monitoring hospitals in Tokyo, the number of patients receiving blood transfusions was calculated to be 1·2 million per year in Japan [27]. The risks of transfusion-transmitted HBV, HCV and HIV infections were estimated at 13–17 cases per year (1 in 70 588–92 307), 1 case every 2–4 years (1 in 2 400 000–4 800 000), and 1 case in 4 years (1 in 4 800 000), respectively. In fact, 74, 41 and 0 cases of HBV, HCV and HIV infections, respectively, associated with transfusion were reported to the JRC in 2007. Investigation of these confirmed transfusion as the cause of 13 cases of HBV and 1 case of HCV [28].

Hollinger [29] has indicated that the reagents used in Japan to test blood donations and the criteria used by the JRC for the release of donations are different from those used in Western countries [30–33]. In Japan, if a specimen is reactive for anti-HBc at a titre of <1:32 based on a hemagglutination inhibition test or is ≥1:32 along with a passive hemagglutination inhibition assay revealing an anti-HBs titre of ≥200 mIU/ml, the blood components can be transfused to patients [3, 4]. We attempted to improve our understanding of the risk of routine transfusion of blood components to patients when these criteria are used. Pre-transfusion specimens, cryopreserved in Osaka City University hospitals, were tested for anti-HBs and anti-HBc. The specimens were non-reactive for HBsAg in all 247 patients tested but were serologically reactive for anti-HBs and/or anti-HBc in 82 patients. The remaining 165 patients whose pre-transfusion specimens were non-reactive for HBsAg, anti-HBc, anti-HBs and HBV DNA were transfused with 686 blood components. The repository specimens of the 686 donors concerned were tested for HBsAg, anti-HBs, anti-HBc and HBV DNA. Specimens of 64 of the donors were reactive for anti-HBs and/or anti-HBc, and their blood components (64) were transfused to 52 patients. None of

the HBV markers changed in those patients receiving these components indicating that the blood components with low anti-HBc and/or high anti-HBs titres and with non reactive results for HBV DNA by MP-NAT have a low risk (rate, 0; 95% confidence interval for the risk of transmission, <1 in 22) (Fig. 2).

The JRC implemented a chemiluminescent EIA system (Fujirebio Inc., Tokyo, Japan) in 2008 replacing the earlier agglutination method. We have continued the same strategy of using blood from donors with low anti-HBc titres (cut-off index <12) or high anti-HBc titres (cut-off index≥12) with high anti-HBs titres (≥200 mIU/ml) for transfusion because discarding these blood components (86/1009, 8·5% in Table 4) would have a huge influence on our ability to maintain a stable blood supply to hospitals. However, most of these donors are ≥50 years in most cases [34], and it is likely that we will be able to review this approach and adopt a policy of only issuing anti-HBc-negative blood components in the future. Meanwhile, we will continue to evaluate the residual risk of blood transfusion from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres.

Collaborating institutes

Asahikawa Medical College Hospital (Hokkaido).
Iwate Medical University Hospital (Iwate).
Osaka City University Hospital (Osaka).
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Fukuoka University Hospital (Fukuoka).

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Conflict of interest

The authors have no conflict of interest to declare regarding this manuscript.

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Original Article

Response-guided therapy for patients with chronic hepatitis who have high viral loads of hepatitis C virus genotype 2

Yasunori Yamaguchi,[1] Akihiro Tamori,[1] Yasuhito Tanaka,[2] Shuji Iwai,[1] Sawako Kobayashi,[1] Hideki Fujii,[1] Hiroyasu Morikawa,[1] Atsushi Hagihara,[1] Masaru Enomoto[1] and Norifumi Kawada[1]

[1]*Department of Hepatology, Osaka City University Graduate School of Medicine, Osaka, and* [2]*Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*

Aim: We evaluated the efficacy of response-guided therapy in patients with hepatitis C virus (HCV) genotype 2.

Methods: We studied 105 patients with an HCV genotype 2 load of higher than 5.0 Log IU/mL who received more than 75% of the target dose of pegylated interferon plus ribavirin. Among patients with rapid viral response (RVR; no HCV RNA detected at week 4), 14 selected 16 weeks of therapy (group A), and 28 selected 24 weeks of therapy (group B). Among non-RVR patients, 40 selected 24 weeks of therapy (group C), and 19 selected 48 weeks of therapy (group D).

Results: All patients in group A and B achieved a sustained viral response (SVR). Clinical characteristics did not differ significantly between groups C and D. However, the proportion of patients in whom HCV RNA disappeared at a later week after starting treatment was higher in group D ($P = 0.0578$). SVR rate was 73% in C, and 79% in D. Among patients in whom HCV RNA disappeared between weeks 5 and 8, SVR was achieved in 28 (82%) of 34 patients in C and 10 (91%) of 11 patients in D. Among patients whose HCV RNA disappeared between weeks 9 and 12, SVR was achieved in one (20%) of five patients in C and five (63%) of eight patients in D (not statistically significant).

Conclusions: 16 weeks of combination therapy could achieve an adequate antiviral effect for RVR patients. Extending therapy could not significantly improve SVR rate in non-RVR patients.

Key words: extended therapy, rapid responder, slow responder

INTRODUCTION

MORE THAN 170 million people worldwide are chronically infected with hepatitis C virus (HCV).[1] HCV is a major factor for liver disease, including hepatocellular carcinoma (HCC), and is one of the most important health problems worldwide.[2,3] The HCV genotype is one of the most important determinants of the response to antiviral therapy.[4,5] In patients with "difficult-to-treat" genotype 1 infection, addition of an inhibitor of HCV nonstructural protein 3/4A protease, such as telaprevir and boceprevir, to pegylated interferon (PEG-IFN) and ribavirin has become approved very recently.[6,7] In contrast, combination with PEG-IFN and ribavirin is still the standard of care (SOC) for patients with genotype 2 or 3, because approximately 80% of such patients achieve a sustained viral response (SVR) after 24 weeks of the SOC treatment.[4,5]

Several randomized controlled trials have suggested that rapid viral responders with genotype 2 achieve an adequate SVR rate after 12 to 16 weeks of combination therapy.[8–10] However, the largest, multicenter, United States-based trial (ACCELERATE) showed that shortening the duration of therapy from 24 to 16 weeks in patients with genotype 2 or 3 lowered the probability of attaining SVR, even in rapid viral responders.[11] Recent

Correspondence: Dr Akihiro Tamori, Department of Hepatology, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan. Email: atamori@med.osaka-cu.ac.jp

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