[21] Kohno N. Serum marker KL-6/MUC1 for the diagnosis and management of interstitial pneumonitis. J Med Invest 1999;46:151–8.
[22] Arase Y, Ikeda K, Tsubota A, et al. Usefulness of serum KL-6 for early diagnosis of idiopathic pulmonary fibrosis in patients with hepatitis C virus. Hepatol Res 2003;27:89–94.
[23] Tokita H, Fukui H, Tanaka A, et al. Circulating KL-6 level at baseline is a predictive indicator for the occurrence of interstitial pneumonia during interferon treatment for chronic hepatitis C. Hepatol Res 2003;26:91–7.
[24] Moriyama M, Matsumura H, Mikuni M, et al. The clinical significance of serum KL-6 levels in patients with type C liver diseases. Hepatol Res 2003;25:385–95.
[25] Moriyama M, Matsumura H, Watanabe A, et al. Detection of serum and intrahepatic KL-6 in anti-HCV positive patients with hepatocellular carcinoma. Hepatol Res 2004;30:24–33.
[26] Ogawa M, Yamamoto T, Kubo S, et al. Clinicopathologic analysis of risk factors for distant metastasis of hepatocellular carcinoma. Hepatol Res 2004;29:228–34.
[27] Tamano M, Sugaya H, Oguma M, et al. Serum and tissue PIVKA-II expression reflect the biological malignant potential of small hepatocellular carcinoma. Hepatol Res 2002;22:261–9.
[28] Aoyagi Y, Mita Y, Suda T, et al. The fucosylation index of serum alpha-fetoprotein as useful prognostic factor in patients with hepatocellular carcinoma in special reference to chronological changes. Hepatol Res 2002;23:287.
[29] Koike Y, Shiratori Y, Sato S, et al. Des-gamma-carboxy prothrombin as a useful predisposing factor for the development of portal venous invasion in patients with hepatocellular carcinoma: a prospective analysis of 227 patients. Cancer 2001;91:561–9.
[30] Hamamura K, Shiratori Y, Shiina S, et al. Unique clinical characteristics of patients with hepatocellular carcinoma who present with high plasma des-gamma-carboxy prothrombin and low serum alpha-fetoprotein. Cancer 2000;88:1557–64.
[31] Igarashi H, Aoyagi Y, Suda T, Mita Y, Kawai K. Studies on the correlation among the fucosylation index, concentration of alpha-fetoprotein and des-gamma-carboxy prothrombin as prognostic indicators in hepatocellular carcinoma. Hepatol Res 2003;27: 280–8.
[32] Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–20.
[33] Fukuzaki T, Kawata S, Imai Y, et al. Changes in serum hepatic fibrosis markers in biochemical responders to interferon therapy for chronic hepatitis C. Hepatol Res 2000;17:156–66.
[34] Kubo S, Tanaka H, Shuto T, et al. Correlation between low platelet count and multicentricity of hepatocellular carcinoma in patients with chronic hepatitis C. Hepatol Res 2004;30:221–5.
[35] Qiu Y, Hoshida Y, Kato N, et al. A simple combination of serum type IV collagen and prothrombin time to diagnose cirrhosis in patients with chronic active hepatitis C. Hepatol Res 2004;30:214–20.
[36] Suzuki K, Takada H, Oka S, et al. Clinical significance of KL-6, a marker of interstitial pneumonia, in cases of HCV-associated chronic liver disease. Intern Med 2003;42:650–4.
[37] Hirasawa Y, Kohno N, Yokoyama A, Inoue Y, Abe M, Hiwada K. KL-6, a human MUC1 mucin, is chemotactic for human fibroblasts. Am J Respir Cell Mol Biol 1997;17:501–7.
[38] Uchida T. Pathology of hepatitis C. Intervirology 1994;37:126–32.
[39] Shibata M, Morizane T, Uchida T, et al. Irregular regeneration of hepatocytes and risk of hepatocellular carcinoma in chronic hepatitis and cirrhosis with hepatitis-C-virus infection. Lancet 1998;351:1773–7.
[40] Ueno Y, Moriyama M, Uchida T, Arakawa Y. Irregular regeneration of hepatocytes is an important factor in the hepatocarcinogenesis of liver disease. Hepatology 2001;33:357–62.
[41] Gendler SJ. MUC1, the renaissance molecule. J Mammary Gland Biol Neoplasia 2001;6:339–53.
[42] Taylor-Papadimitriou J, Burchell JM, Plunkett T, et al. MUC1 and the immunobiology of cancer. J Mammary Gland Biol Neoplasia 2002;7:209–21.
[43] Ringel J, Lohr M. The MUC gene family: their role in diagnosis and early detection of pancreatic cancer. Mol Cancer 2003;2:9.
[44] Li Y, Kufe D. The Human DF3/MUC1 carcinoma-associated antigen signals nuclear localization of the catenin p120(ctn). Biochem Biophys Res Commun 2001;281:440–3.
[45] Yamamoto M, Bharti A, Li Y, Kufe D. Interaction of the DF3/MUC1 breast carcinoma-associated antigen and beta-catenin in cell adhesion. J Biol Chem 1997;272:12492–4.
[46] Ren J, Li Y, Kufe D. Protein kinase C delta regulates function of the DF3/MUC1 carcinoma antigen in beta-catenin signaling. J Biol Chem 2002;277:17616–22.
[47] Li Y, Ren J, Yu W, et al. The epidermal growth factor receptor regulates interaction of the human DF3/MUC1 carcinoma antigen with c-Src and beta-catenin. J Biol Chem 2001;276:35239–42.
[48] Pandey P, Kharbanda S, Kufe D. Association of the DF3/MUC1 breast cancer antigen with Grb2 and the Sos/Ras exchange protein. Cancer Res 1995;55:4000–3.
[49] Li Y, Chen W, Ren J, et al. DF3/MUC1 signaling in multiple myeloma cells is regulated by interleukin-7. Cancer Biol Ther 2003;2:187–93.
[50] Li Y, Kuwahara H, Ren J, Wen G, Kufe D. The c-Src tyrosine kinase regulates signaling of the human DF3/MUC1 carcinoma-associated antigen with GSK3 beta and beta-catenin. J Biol Chem 2001;276:6061–4.
[51] Wen Y, Caffrey TC, Wheelock MJ, Johnson KR, Hollingsworth MA. Nuclear association of the cytoplasmic tail of MUC1 and beta-catenin. J Biol Chem 2003;278:38029–39.
[52] Kudo M, Chung H, Osaki Y. Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). J Gastroenterol 2003;38:207–15.
[53] Hughes OD, Bishop MC, Perkins AC, et al. Targeting superficial bladder cancer by the intravesical administration of copper-67-labeled anti-MUC1 mucin monoclonal antibody C595. J Clin Oncol 2000;18:363–70.
[54] Apostolopoulos V, Pietersz GA, McKenzie IF. MUC1 and breast cancer. Curr Opin Mol Ther 1999;1:98–103.

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HEPATOLOGY

Mutations in the NS5B region of the hepatitis C virus genome correlate with clinical outcomes of interferon-alpha plus ribavirin combination therapy

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Abstract

Background and Aim: Combination treatments of interferon-alpha (IFN) and ribavirin (RBV) are more effective than those of IFN alone in hepatitis C virus (HCV) infection. However, mechanisms of the action of the combination regimen are not well understood. To elucidate the viral genetic basis of IFN plus RBV combination therapy, genetic variabilities of HCV-1b were analyzed.

Methods: We performed pair-wise comparisons of full-length HCV genomic sequences in three patients' sera before and after initiation of IFN plus RBV treatment. Subsequently, we analyzed amino acid sequences of the NS5B region, which codes for the viral RNA-dependent RNA polymerase, and compared these with the outcomes of the therapy in 81 patients.

Results: Analysis of the entire HCV sequence in patients who received IFN plus RBV therapy did not show consistent amino acid changes between before and after the initiation of the therapy. NS5B sequence analyses revealed that mutations at positions 300–358 of NS5B, including polymerase motif B to E, occurred more frequently in a group of patients exhibiting a sustained viral response (SVR) or an end-of-treatment response (ETR) compared with a group of patients exhibiting a non-response (NR). Closer examination revealed that mutations at aa 309, 333, 338 and 355 of NS5B occurred significantly more frequently in the SVR plus ETR group than in the NR group ($P = 0.0004$). Multivariate analysis showed that the number of mutations at these four sites was an independent predictor of SVR plus ETR *versus* NR.

Conclusions: Particular amino acid changes in the NS5B region of HCV may correlate with outcomes of IFN plus RBV combination therapy.

Key words: amino acid sequence, error catastrophe, RNA-dependent RNA polymerase, transition.

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, which can lead to liver cirrhosis and hepatocellular malignancy.[1,2] Interferon (IFN) is the agent of choice for treating HCV infection. However, IFN monotherapy produces sustained virological responses in only 15–20% of patients treated, most of whom relapse after completion of the therapy.[3,4] Several recent studies of combination therapy with IFN alpha 2b and ribavirin (RBV) have shown that the regimen induces higher sustained virological responses than IFN monotherapy. Unfortunately, 50–60% of patients still do not respond to the combination therapy.[5–8]

RBV is a synthetic guanosine analog with broad antiviral actions *in vitro* against various DNA and RNA

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viruses.[9,10] At present, four mechanisms of action have been postulated: (i) immune modulatory effects by a switching of T-cell phenotype from Th2 to Th1 that induces type 1 cytokine responses;[11-13] (ii) inhibition of inosine monophosphate dehydrogenase (IMPDH) leading to depletion of cellular GMP pool;[14] (iii) mutagenic activity against RNA viruses that induces misincorporation of RBV triphosphate into viral RNA leading to error prone replication of viral genome;[15-18] and (iv) inhibition of the activity of HCV NS5B RNA-dependent RNA polymerase (RdRp).[19,20] However, it has not been fully understood which mechanisms of actions of RBV are effective against HCV infection.

Certain genetic structures of viruses may affect the sensitivity to their therapeutic drugs. Nucleoside analogs are widely used against viruses such as human immunodeficiency virus type 1 (HIV) and hepatitis B virus (HBV).[21,22] The antiviral effect of those reagents arises from the inhibition of viral DNA/RNA polymerase activity. However, single or multiple mutation(s) in the viral polymerase confer drug resistance and help the drug resistant strains emerge.[22-30] Also in HCV infection, the INF sensitivity determining region (ISDR) of HCV genome, which we have previously identified, critically determines the virological response to IFN and the treatment outcomes.[31,32] As to RBV, one study of five HCV genotype 1a patients who had undergone RBV monotherapy has reported one mutation in NS5B that may correlate with RBV sensitivity.[33] These findings make us speculate that genetic variability of HCV NS5B region, which codes for RdRp, may correlate with sensitivity to RBV and may influence the outcomes of IFN plus RBV combination therapy.

In the present study, we first analyzed effects of RBV on HCV genomic structure and the viral genetic basis of RBV resistance by performing pair-wise comparisons of full-length HCV genomic sequences in patient sera before and after initiation of IFN plus RBV treatment. Subsequently, we have investigated a hypothesis that genomic variability of HCV RdRp may confer resistance or susceptibility to RBV and may correlate with the outcomes of IFN plus RBV combination therapy. Thus, we analyzed amino acid sequences of the NS5B region and the outcomes of IFN plus RBV combination therapy in 81 patients, and found that certain amino acid variations in the NS5B region may associate with the treatment outcomes.

METHODS

Patients of interferon plus ribavirin non-responders

Three patients infected with HCV, genotype 1b, were studied. All patients were non-responders to combination therapy with IFN alfa-2b (Intron A, Schering Plough, Kenilworth, NJ, USA), 6 million units three times per week plus RBV (Rebetoron, Schering Plough), 800 mg/day (> 12.1 mg/kgBW) for 24 weeks. Serum samples were obtained before treatment and at 12 weeks after initiation of the treatment, and pair-wise comparisons of the consensus sequences of full-length HCV genomes were performed. As controls for the IFN plus RBV therapy data, we analyzed our previously published HCV sequence data for three non-responders of IFN monotherapy[32] (deposited with the DDBJ/GenBank/EMBL data libraries under accession number D50483, D50480, D50485, D50481, D50484 and D50482).

RNA extraction, reverse transcription-polymerase chain reaction and direct sequencing

RNA was extracted from patient sera by the modified acid guanidinium thiocyanate-phenol-chloroform (AGPC) method,[34] using ISOGEN reagent (Wako Pure Chemical Industries, Osaka, Japan), and reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described.[32] Full-length HCV genomes were amplified by nested PCR with 21 partially overlapping sets of primers, as previously reported.[32] M13-forward and M13-reverse sequencing primer sequences were attached to the 5′-termini of sense and antisense nested PCR primers. Each PCR product was purified by a spin filtration column (Suprec-02; Takara). Both strands of the PCR products were cycle sequenced with the PRISM dye termination kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions, and consensus nucleotide sequences were determined by an automated DNA sequencer model 373 A (Applied Biosystems).

Sequence analyses

Nucleotide sequencing analysis was performed with a software program (MEGA version 2.1) to calculate values for d_N (non-synonymous substitution), d_S (synonymous substitution), d_N/d_S ratios, and the number of point mutations.

Clinical outcome of combination therapy

Patients were placed into one of three outcome groups.

- Sustained virologic response (SVR): HCV-RNA was not detectable by RT-PCR for 6 months following completion of the therapy.
- End-of-treatment response (ETR): HCV-RNA was not detected at the end of the treatment, but reappeared within 6 months thereafter.
- Non-response (NR): HCV-RNA did not disappear during the treatment.

Nucleoside sequencing analyses of the NS5b region

Amino acid mutations in the conserved motifs (motif A, B, C, D, E, F)[35-38] in NS5B RdRp were retrospectively analyzed in 81 HCV genotype 1b patients who were

treated with IFN alfa-2b, 6 million units three times per week plus RBV, 800 mg/day (> 12.1 mg/kgBW) for 24 weeks. All patients had biopsy-proven chronic hepatitis with positive serum HCV antibodies and serum HCV-RNA. RNA was extracted from sera of the patients before treatment. NS5B region, including motifs A to F, was amplified by RT-PCR and sequences corresponding to nucleotides 7730–8874 of HCV-J were determined.[32] The deduced amino acid sequences of all patients were aligned and compared with consensus sequences for mutations and analyzed for correlation between amino acid mutations of NS5B and the clinical outcome of the combination therapy.

Statistical analyses

Comparisons of differences in categorical data between groups were performed using the χ^2 test and Fishers exact test. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test for two groups and by the Kruskal–Wallis test or Scheffé method for three groups. Multivariate analysis was carried out by multiple logistic regression analysis. *P*-values of less than 0.05 were defined as statistically significant.

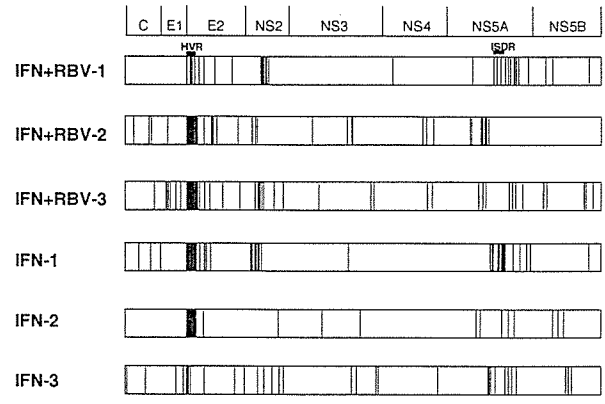

Figure 1 Schematic representation of the distribution of mutations in amino acid residues during the combination therapy and interferon (IFN) monotherapy. Distributions of amino acid changes in the entire hepatitis C virus (HCV) genome in patient serum before treatment and 12 weeks after initiation of treatment are shown. The upper three data are from patients treated with IFN/ribavirin (RBV) combination therapy (IFN + RBV 1–3), and the lower three data are those treated with IFN monotherapy (IFN 1–3). Vertical lines in each HCV polyproteins show position of amino acid differences during the therapy.

RESULTS

Pair-wise comparisons of the full-length HCV genome in three patients before and after initiation of IFN/RBV treatment

HCV genomes from the three study patients comprised 9423 nucleotides and contained an open reading frame of 3010 amino acids. In patient one, 31 amino acid changes were found in the HCV genome. These amino acid changes were clustered in the E2-hypervariable regions (8 of 31) and the NS5A regions (11 of 31). Before treatment, the INF-sensitivity determining lesion (ISDR)[31,32] were 'mutant' type with five amino acid changes compared with consensus sequence, which changed to 'intermediate' type with two amino acid changes after the initiation of treatment. In patient two, 37 amino acid changes were found in the entire HCV genome. The changes were exclusively found in the E2-hypervariable region (16 out of 37 amino acids), while there was no change in the ISDR. In patient three, 56 amino acid changes were found. The changes were exclusively found in the E2 region (24 out of 56 amino acids). Distribution of amino acid changes during the therapy in the three patients treated with combination therapy and three non-responders to IFN monotherapy are illustrated in Figure 1. The numbers of nucleotide changes for the three study patients were 88, 130 and 272, respectively. The d_N/d_S ratios were 0.195, 0.148 and 0.099, respectively. Among the three control subjects who received IFN monotherapy, the numbers of nucleotide changes were 138, 160 and 175, respectively. The d_N/d_S ratios were 0.158, 0.061 and 0.089, respectively. As shown in Figure 2, d_N/d_S ratios tended to be higher in the E2 region than in the other regions during

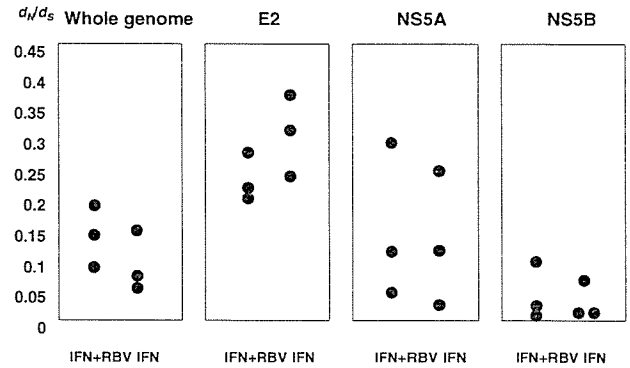

Figure 2 Ratio of non-synonymous to synonymous distances for the E2, NS5A, NS5B and whole hepatitis C virus (HCV) genome. The d_N/d_S ratio in E2 region tended to be higher than other regions during interferon (IFN) monotherapy and during combination therapy. All pairwise d_N/d_S ratios were calculated using MEGA version 2.1 for each subject.

both IFN monotherapy and combination therapy. The numbers of transitional mutations in patients who received the combination therapy had 71 (80.2% of total mutations), 104 (80.0%) and 218 (80.1%) transitional mutations, respectively, and in patients who received IFN monotherapy these were 108 (78.3%), 131 (81.9%) and 130 (75.4%), respectively. The proportion of transitions among IFN monotherapy patients did not differ from the proportion among combination therapy patients.

Two studies have observed two key transitions, C-to-U and G-to-A, in genomic sequences of RBV-treated RNA viruses.[17,18] In the present study, C-to-U and G-to-A mutations comprised 35.5%, 40.6% and 58% of

total mutations, respectively, in the three patients treated with IFN monotherapy, and 43.2%, 38.3% and 37.8%, respectively, in those treated with combination therapy. These results showed no obvious increase in key mutations of C-to-U and G-to-A associated with the combination therapy (Table 1).

Sequence analyses of NS5b region in 81 patients treated with IFN and RBV therapy

To study the correlation between the genetic structures of NS5B and the outcome of IFN plus RBV combination therapy, amino acid sequences of HCV NS5B (aa. 61–407), including motif A-F, were analyzed in 81 patients treated with IFN plus RBV combination therapy. The clinical characteristics of the patients are shown in Table 2. Nineteen (23.5%) patients were SVR, 40 (49.4%) were ETR, and 22 (27.2%) were NR. Clinical variables were analyzed according to the results of the combination therapy. Univariate analysis identified fibrosis stage as significantly lower in the SVR patients than in the other patients. No other clinical parameters were significantly correlated with the responses.

The amino acid sequences of the essential motif B to E of NS5B in these 81 patients are aligned with consensus sequences in Figure 3. Comparison of the NS5B sequences between patients with SVR and patients with non-SVR (ETR and NR) showed no obvious differences. Instead, when we compared the sequences of a patient group of SVR plus ETR with those of patients with NR, the mutations at position NS5B 300–358, including motif B to E between, were more frequent in the SVR plus ETR group than in the NR group. When we analyzed mutations of individual amino acid positions, the frequencies of mutations at aa 309, 333, 338 and 355 of NS5B (the four sites) were found to be more frequent in patients with SVR or ETR than those with NR (Fig. 4). The total number of amino acid changes at these four sites was significantly higher in patients with SVR or ETR than those with NR (0.93 ± 0.89 *vs* 0.27 ± 0.70, $P = 0.0004$). In 19 SVR patients, five patients had no mutations, 10 patients had one mutation, and four patients had two or more mutations at the four sites. In the 40 ETR patients, 18 patients had no mutations, 13 patients had one mutation, and nine patients had two or more mutations at the four sites. In 22 NR patients, 19 patients had no mutations, two patients had one mutation, and one patient had three mutations at the four sites (Fig. 5a). The SVR rates were 11.9% (5 of 42) and 35.9% (14 of 39) in patients who had none and one or more mutations at the four sites, respectively (Fig. 5b). Patients with increased mutations at the four sites tended to be in the SVR or ETR groups. We subsequently analyzed various clinical factors by multivariate analysis among the three response groups to determine the independent predictors for SVR and NR (Table 3). Among these clinical factors, the NS5B mutation described above was independently associated with NR ($P = 0.0185$).

Mutations of the NS5B region, which codes for the viral RdRp, may alter its enzymatic activities which may influence serum virus load of each patient. In our results, however, there was no obvious correlation between the number of NS5B mutations and serum viral loads in each patient, nor was there a difference in the serum virus loads between the patient groups categorized by the numbers of mutations at aa 309, 333, 338 and 355 of NS5B.

Table 1 Sequence analysis of full genome of hepatitis C virus (HCV) RNA treated with interferon (IFN) plus ribavirin

	G-to-A and C-to-U	Other transition (A-to-G and U-to-C)
IFN plus ribavirin	58.3	72.5
No ribavirin (IFN monotherapy)	60.8	70.4

Mutations per 10 000 nucleotides. A total of 56 538 nucleotides were sequenced.

DISCUSSION

In the present study, we have demonstrated that particular amino acid changes in the NS5B region of HCV

Table 2 Baseline characteristics of the group of 81 patients, segregated according to the clinical outcome of interferon (IFN) plus ribavirin combination therapy

	SVR	ETR	NR	*P*-value
Number of patients	19	40	22	
Age (years)	49.5 ± 12.2	55.9 ± 8.1	57.2 ± 10.6	NS
Sex (male/female)	15/4	27/13	11/11	NS
Baseline ALT (IU/L)	122.2 ± 88.0	80.2 ± 43.2	107.4 ± 73.7	NS
Platelet count (10^3/mm^3)	16.0 ± 5.5	16.3 ± 5.5	14.7 ± 4.5	NS
Fibrosis stage (SD)	1.41 ± 0.71	1.92 ± 0.94	2.10 ± 0.72	0.012†
Serum HCV RNA at baseline (KIU/mL)	480.5 ± 295.7	594.6 ± 239.3	599.9 ± 271.3	NS
Number of ISDR mutations	1.73 ± 2.92	0.80 ± 1.22	1.00 ± 1.80	NS

†Significant differences between SVR and others. Values are expressed as mean ± SD, except where noted. ALT, alanine aminotransferase; ETR, end-of-treatment responder; NR,-non-responder; NS, not significant; SR, sustained responder.

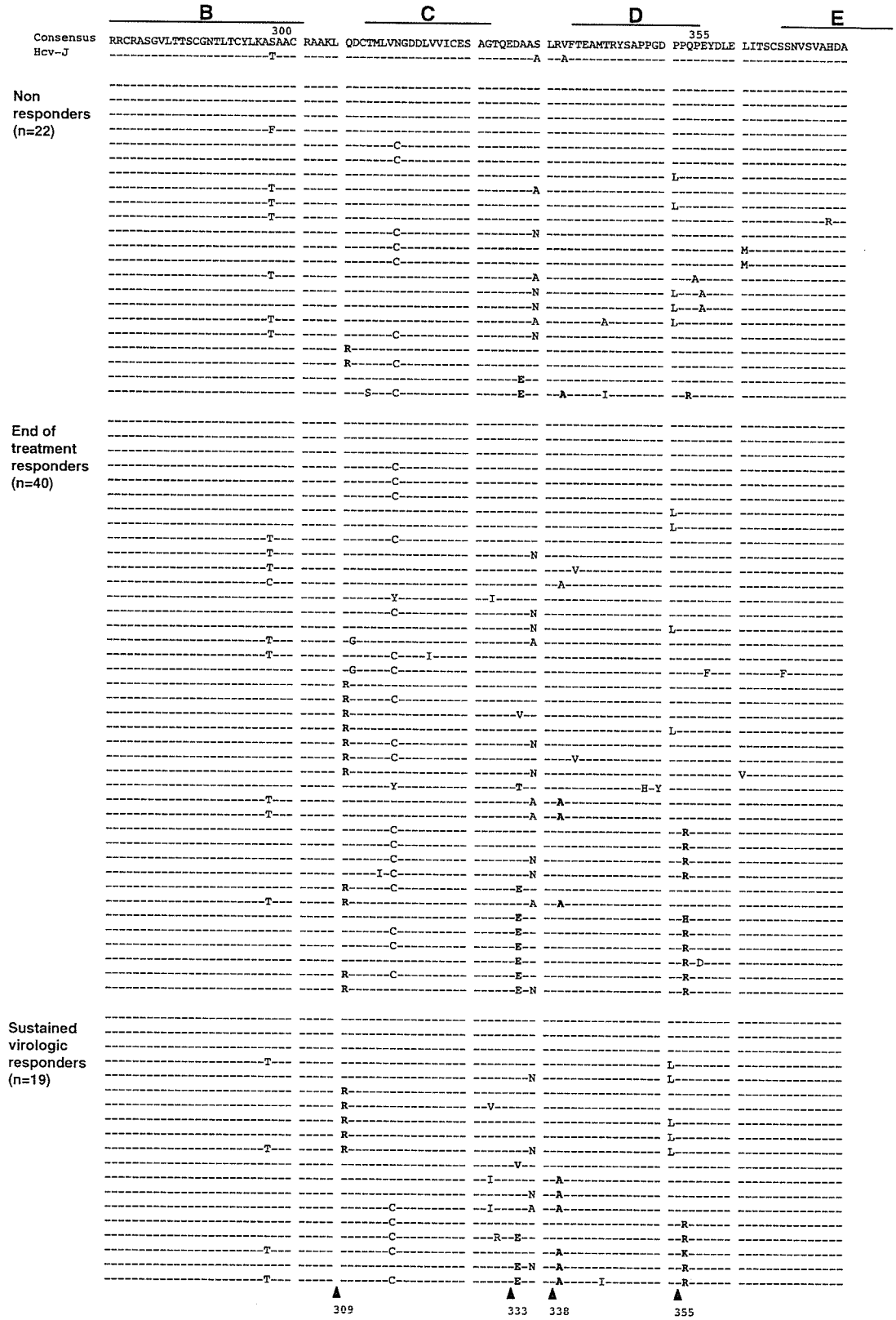

Figure 3 Amino acid sequence alignments of the NS5B motif B-E in 81 patients with chronic hepatitis C virus (HCV)-1b infection and treated with interferon (IFN) plus ribavirin for 24 weeks. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate the identical amino acid residues with consensus sequence and HCV-J shown at the top. At four amino acid positions (NS5B 309, 333, 338 and 355), usages of amino acid residues differ between non-responders (NR) and others. Changes of this part are indicated by bold letter. Outcome of combination therapy is shown on the left side.

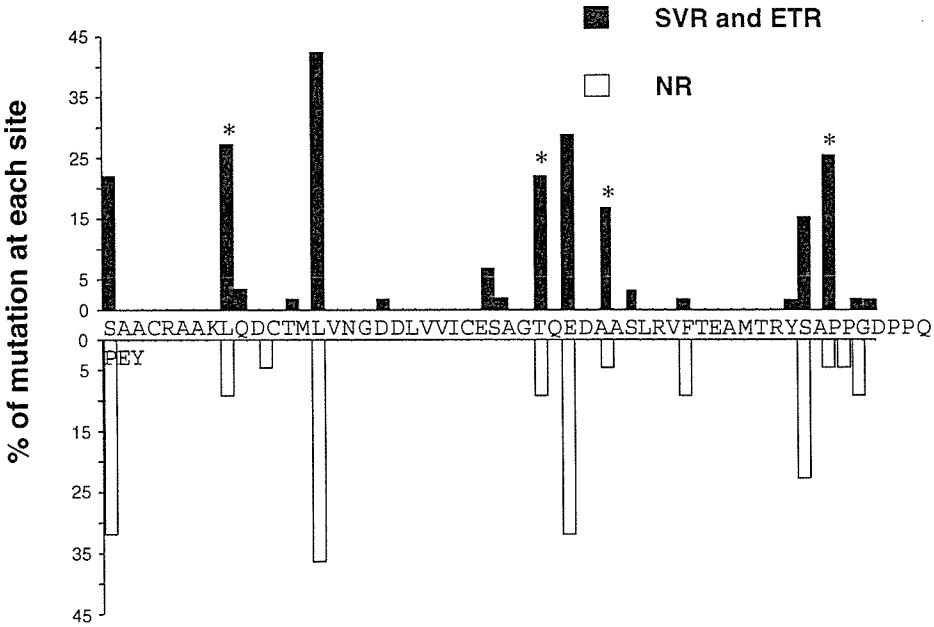

Figure 4 Relationship between frequency of mutations at each site in NS5B 300–358 and the efficacy of interferon (IFN) plus ribavirin treatment. Amino acid residues are indicated by the standard single-letter codes. Among these 59 sites, mutations of aa NS5B 309, 333, 338 and 355 (identified by *) are frequent in sustained virologic response (SVR) and end-of-treatment response (ETR) patients. NR, non-response.

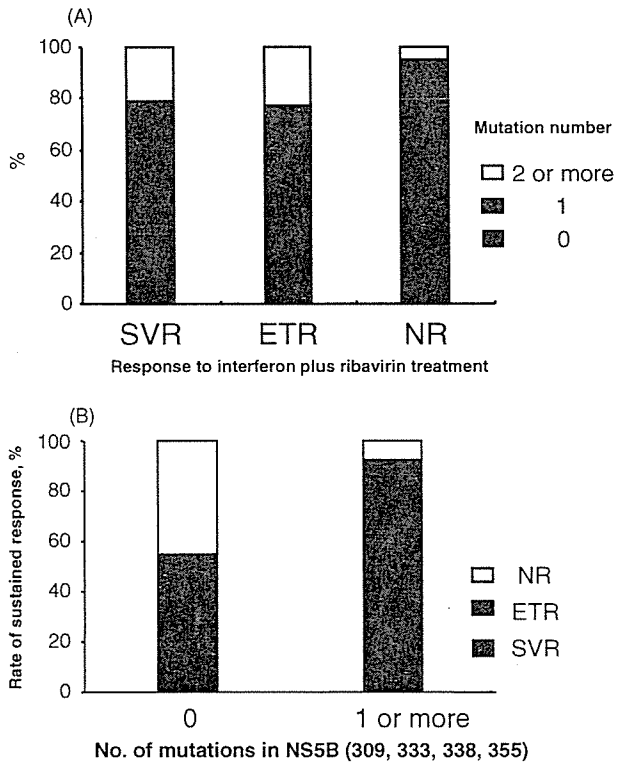

Figure 5 Relationship between number of mutations in NS5B 309, 333, 338, 355 and the outcome of interferon (IFN) plus ribavirin treatment. (a) Distribution of total numbers of mutations at aa. 309, 333, 338 and 355 of NS5B according to sustained virologic response (SVR), end-of-treatment response (ETR) and non-response (NR) patients. (b) Proportion of SVR, ETR and NR patients between groups with or without mutations at aa. 309, 333, 338 and 355 of NS5B.

Table 3 Multivariate analysis for the clinical and virological factors affecting virological responses (SVR and NR) to interferon (IFN) plus ribavirin combination therapy in the group of 81 patients

	Patient with SVR *P*-value	Patient with NR *P*-value
Age (years)	0.572	0.598
Sex (male/female)	0.814	0.158
Baseline ALT (IU/L)	0.022	0.981
Platelet count (10^3/mm^3)	0.749	0.627
Mean fibrosis stage (SD)	0.037	0.330
Serum HCV RNA at baseline	0.227	0.890
No. of ISDR mutations	0.491	0.754
No. of NS5B mutations (309,333,338,355)	0.057	0.019

ALT, alanine aminotransferase; ETR, end-of-treatment response; ISDR, interferon sensitivity determining region; NR, non-response; SR, sustained response.

correlate with the clinical outcome of combination therapy. Pair-wise comparisons of the full-length HCV genome in three patient sera obtained before and 12 weeks after the start of IFN plus RBV therapy did not show consistent amino acid changes. The results suggest negative evidence against the presence of treatment-resistant viral sub-populations. On the contrary, subsequent analyses of mutation patterns in the NS5B region in 81 patients showed a significant correlation between particular amino acid mutations of NS5B and the outcome of the combination therapy. Mutations of aa. 309, 333, 338 and 355 of the NS5B were significantly more frequent in SVR and ETR patients, in which the virus has been persistently or at least tempo-

rarily eliminated. Total numbers of mutations at the four amino acid positions were significantly more in SVR and ETR patients compared to NR patients (0.93 ± 0.89 *vs* 0.27 ± 0.70; $P = 0.0004$). These data suggest that particular amino acid mutations of NS5B-RdRp protein may confer sensitivity to combination therapy.

Recently, several studies on mutational analyses of HCV NS5B have identified several key residues responsible for its RdRp activity. Lohmann *et al.* noted that one single amino acid substitution in NS5B increased the efficacy of colony formation by 500-fold in HCV subgenomic replicon.[39] Cheney *et al.* noted that several amino acid substitutions (K155A, R168A, D225N and R386Q) were detrimental to both *in vitro* polymerase activity and replicon RNA replication in Huh-7 cells.[40] Recently, Young *et al.* suggested that NS5B F415Y mutation in HCV-1a was a key resistant variant for RBV monotherapy.[33] However, Y415 is the consensus residue for all genotypes except for 1a and 6a. In the present study of three non-responders, there was no difference at NS5B Y415 between sera collected before treatment and sera collected 12 weeks after the start of treatment with combination therapy.

The locations of the four mutations within the calculated tertiary structure of NS5B RdRp are illustrated in Figure 6a,b. The mutations in NS5B, which were more frequently found in the SVR and the ETR patients, were clustered in motif B to E of RdRp. The amino acid 309 and 355 are both located on the enzyme surface of the substrate entry site. NS5B 333 and 338 are adjacent to the NTP tunnel (Fig. 6b). Because mutations found in HBV and HIV DNA polymerase/reverse transcriptase are known to be located on the surface of the catalytic domain, the mutations in HCV RdRp that were found in the present study may considerably affect their enzymatic activity. Our preliminary data have shown that the HCV subgenomic replicon carrying point mutations in aa. 141 in NS5B less efficiently than the original sequences. Further studies are needed to clarify the role of these point mutations in NS5B in determining the activity of RdRp.

A recent study by Crotty *et al.* has shown that direct antireplicative effects of RBV on viruses include 'error catastrophe' theory in which misincorporations of RBV triphosphate into the viral genome lead to accumulation of mutations in the viral genome and yield defective virus genome. Characteristic pattern of nucleotide mutations by RBV are an increase of G-to-A and C-to-U transition mutations.[17,18] In our present study, although the majority of the mutations were transitions, there was no significant difference in the ratios of the G-to-A and C-to-U mutations between IFN monotherapy and combination therapy (Table 1). One explanation for the discrepancy is that the concentration of RBV in clinical use is too low to act as a mutagen. The clinically achievable blood concentration of RBV is 10–30 μM.[41] On the contrary, an *in vitro* study of polio virus has shown that RBV concentration of 100 μM is required to increase the mutation frequency by at least 1.2-fold.[17] Highly mutated HCV can be excluded or escape detection by RT-PCR and minor clone of HCV quasi-species are excluded by direct sequence of nested PCR products. Therefore, although it is not clear whether RBV is a mutagen against viral genome, our results suggest other mechanisms of RBV contribute to suppress HCV replication, such as inhibition of enzymatic activities of viral RNA polymerase.

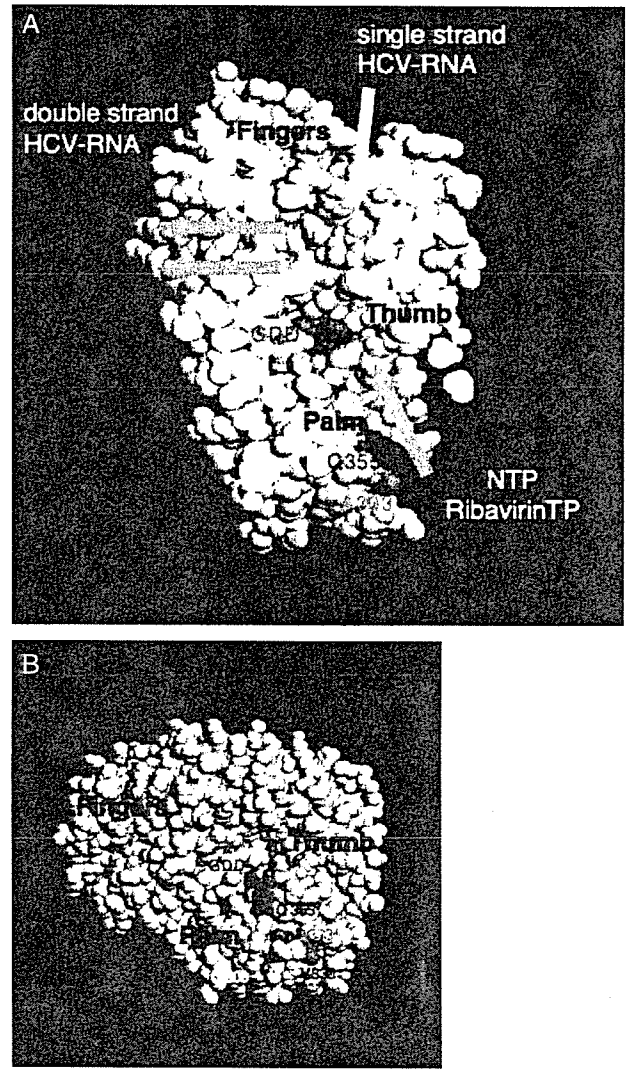

Figure 6 Crystal structure of the hepatitis C virus (HCV) NS5B-RNA dependent RNA polymerase (RdRp). The molecular model of NS5B was constructed using 1QUV from Protein Data Bank (PDB). A space-filling representation of each atom is shown. Graphics were generated using Rasmol 2.7.2.1. (a) Cross-section of the RdRp at level of nucleotide tunnels. The single stranded HCV RNA enters the enzyme through a groove at the top of the finger domain, and the NTP or ribavirin enters the enzyme through the right lower dNTP tunnel (between β fingers and thumb). The essential GDD motif is shown in pink. NS5B 309, 333, 338 and 355 are shown in yellow, orange, green and red, respectively. (b) View from the dNTP entry site.

Many studies have endeavored to identify factors predictive of the outcome of IFN plus RBV combination therapy. Factors that have been examined include pretreatment clinical parameters such as baseline viral load, degree of fibrosis, and gender.[42] One study has

found early viral response (two-log decline of HCV RNA) to be predictive of SVR.[43] Another study showed that ISDR mutations were correlated with the SVR in chronic HCV 1b infection in Taiwan.[44] In the present study, multivariate analysis identified baseline ALT and the degree of fibrosis as independent factors for SVR. Further multivariate analysis showed that the number of mutations at positions NS5B 309, 333, 338 and 355 were independently associated with NR ($P = 0.0185$). The possible implications of our results are that the number of the above-described NS5B mutations is an independent predictive factor and that the parameter predicts NR patients exclusively from SVR or ETR patients. Our results which may enable prediction of NR before initiation of therapy might be of value when we consider indication for IFN plus RBV antiviral therapy or when making a decision about early cessation of the therapy, which may avoid possible side-effects and therapy costs. Although further studies of a larger population of patients are needed, the mutation number might be used to tailor therapy and is a useful factor for clinicians in making a clinical decision to stop treating HCV infection with combination therapy.

Given the absence of proven anti-HCV agents other than IFN and RBV, these combinations will continue to dominate therapy against HCV. Our present results provide evidence of a significant correlation between the response to IFN plus RBV combination therapy in patients with chronic HCV-1b infection and the amino acid changes that were present before therapy in conserved regions of NS5B. Certain amino acid changes in the HCV NS5B-RdRp domain may correlate with the clinical outcome of combination therapy and could thus be an initial predictor for response to IFN plus RBV combination therapy.

REFERENCES

1 Seeff LB, Hoofnagle JH. Appendix: The National Institutes of Health Consensus Development Conference Management of Hepatitis C 2002. *Clin. Liver Dis.* 2003; 7: 261–87.

2 Hoofnagle JH. Course and outcome of hepatitis C. *Hepatology* 2002; **36**: S21–9.

3 Hoofnagle JH, di Bisceglie AM. The treatment of chronic viral hepatitis. *N. Engl. J. Med.* 1997; **336**: 347–56.

4 Poynard T, Bedossa P, Chevallier M *et al.* A comparison of three interferon alfa-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. Multicenter Study Group. *N. Engl. J. Med.* 1995; **332**: 1457–62.

5 Poynard T, Marcellin P, Lee SS *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; **352**: 1426–32.

6 Reichard O, Norkrans G, Fryden A, Braconier JH, Sonnerborg A, Weiland O. Randomised, double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. The Swedish Study Group. *Lancet* 1998; **351**: 83–7.

7 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N. Engl. J. Med.* 1998; **339**: 1485–92.

8 Davis GL, Esteban-Mur R, Rustgi V *et al.* Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. Interventional Hepatitis Interventional Therapy Group. *N. Engl. J. Med.* 1998; **339**: 1493–9.

9 Witkowski JT, Robins RK, Sidwell RW, Simon LN. Design, synthesis, and broad spectrum antiviral activity of 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide and related nucleosides. *J. Med. Chem.* 1972; **15**: 1150–4.

10 Sidwell RW, Huffman JH, Khare GP, Allen LB, Witkowski JT, Robins RK. Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. *Science* 1972; **177**: 705–6.

11 Cramp ME, Rossol S, Chokshi S, Carucci P, Williams R, Naoumov NV. Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. *Gastroenterology* 2000; **118**: 346–55.

12 Tam RC, Lim C, Bard J, Pai B. Contact hypersensitivity responses following ribavirin treatment in vivo are influenced by type 1 cytokine polarization, regulation of IL-10 expression, and costimulatory signaling. *J. Immunol.* 1999; **163**: 3709–17.

13 Ning Q, Brown D, Parodo J *et al.* Ribavirin inhibits viral-induced macrophage production of TNF, IL-1, the procoagulant fgl2 prothrombinase and preserves Th1 cytokine production but inhibits Th2 cytokine response. *J. Immunol.* 1998; **160**: 3487–93.

14 Streeter DG, Witkowski JT, Khare GP *et al.* Mechanism of action of 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. *Proc. Natl. Acad. Sci. USA* 1973; **70**: 1174–8.

15 Severson WE, Schmaljohn CS, Javadian A, Jonsson CB. Ribavirin causes error catastrophe during Hantaan virus replication. *J. Virol.* 2003; 77: 481–8.

16 Contreras AM, Hiasa Y, He W, Terella A, Schmidt EV, Chung RT. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. *J. Virol.* 2002; 76: 8505–17.

17 Crotty S, Cameron CE, Andino R. RNA virus error catastrophe: direct molecular test by using ribavirin. *Proc. Natl. Acad. Sci. USA* 2001; **98**: 6895–900.

18 Crotty S, Maag D, Arnold JJ *et al.* The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. *Nat. Med.* 2000; **6**: 1375–9.

19 Tam RC, Lau JY, Hong Z. Mechanisms of action of ribavirin in antiviral therapies. *Antivir. Chem. Chemother.* 2001; **12**: 261–72.

20 Maag D, Castro C, Hong Z, Cameron CE. Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. *J. Biol. Chem.* 2001; **276**: 46094–8.

21 Hirsch MS, D'Aquila RT. Therapy for human immunodeficiency virus infection. *N. Engl. J. Med.* 1993; **328**: 1686–95.

22 Dienstag JL, Schiff ER, Wright TL *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. *N. Engl. J. Med.* 1999; **341**: 1256–63.

23 Arts EJ, Quinones-Mateu ME, Albright JL *et al.* 3'-Azido-3'-deoxythymidine (AZT) mediates cross-resistance to

nucleoside analogs in the case of AZT-resistant human immunodeficiency virus type 1 variants. *J. Virol.* 1998; **72**: 4858–65.

24 Benhamou Y, Bochet M, Thibault V *et al.* Long-term incidence of hepatitis B virus resistance to lamivudine in human immunodeficiency virus-infected patients. *Hepatology* 1999; **30**: 1302–6.

25 de Jong MD, Veenstra J, Stilianakis NI *et al.* Host-parasite dynamics and outgrowth of virus containing a single K70R amino acid change in reverse transcriptase are responsible for the loss of human immunodeficiency virus type 1 RNA load suppression by zidovudine. *Proc. Natl. Acad. Sci. USA* 1996; **93**: 5501–6.

26 Gauthier J, Bourne EJ, Lutz MW *et al.* Quantitation of hepatitis B viremia and emergence of YMDD variants in patients with chronic hepatitis B treated with lamivudine. *J. Infect. Dis.* 1999; **180**: 1757–62.

27 Imamichi T, Berg SC, Imamichi H *et al.* Relative replication fitness of a high-level 3'-azido-3'-deoxythymidine-resistant variant of human immunodeficiency virus type 1 possessing an amino acid deletion at codon 67 and a novel substitution (Thr-->Gly) at codon 69. *J. Virol.* 2000; **74**: 10958–64.

28 Miller V, Ait-Khaled M, Stone C *et al.* HIV-1 reverse transcriptase (RT) genotype and susceptibility to RT inhibitors during abacavir monotherapy and combination therapy. *Aids* 2000; **14**: 163–71.

29 Shah FS, Curr. KA, Hamburgh ME *et al.* Differential influence of nucleoside analog-resistance mutations K65R and L74V on the overall mutation rate and error specificity of human immunodeficiency virus type 1 reverse transcriptase. *J. Biol. Chem.* 2000; **275**: 27037–44.

30 Winters MA, Shafer RW, Jellinger RA, Mamtora G, Gingeras T, Merigan TC. Human immunodeficiency virus type 1 reverse transcriptase genotype and drug susceptibility changes in infected individuals receiving dideoxyinosine monotherapy for 1–2 years. *Antimicrob. Agents Chemother.* 1997; **41**: 757–62.

31 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N. Engl. J. Med.* 1996; **334**: 77–81.

32 Enomoto N, Sakuma I, Asahina Y *et al.* Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J. Clin. Invest.* 1995; **96**: 224–30.

33 Young KC, Lindsay KL, Lee KJ *et al.* Identification of a ribavirin-resistant NS5B mutation of hepatitis C virus during ribavirin monotherapy. *Hepatology* 2003; **38**: 869–78.

34 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 1987; **162**: 156–9.

35 Poch O, Sauvaget I, Delarue M, Tordo N. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J.* 1989; **8**: 3867–74.

36 Lesburg CA, Cable MB, Ferrari E, Hong Z, Mannarino AF, Weber PC. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat. Struct. Biol.* 1999; **6**: 937–43.

37 Lohmann V, Korner F, Herian U, Bartenschlager R. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J. Virol.* 1997; **71**: 8416–28.

38 Lohmann V, Roos A, Korner F, Koch JO, Bartenschlager R. Biochemical and structural analysis of the NS5B RNA-dependent RNA polymerase of the hepatitis C virus. *J. Viral Hepat.* 2000; **7**: 167–74.

39 Lohmann V, Korner F, Dobierzewska A, Bartenschlager R. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J. Virol.* 2001; **75**: 1437–49.

40 Cheney IW, Naim S, Lai VC *et al.* Mutations in NS5B polymerase of hepatitis C virus: impacts on in vitro enzymatic activity and viral RNA replication in the subgenomic replicon cell culture. *Virology* 2002; **297**: 298–306.

41 Larrat S, Stanke-Labesque F, Plages A, Zarski JP, Bessard G, Souvignet C. Ribavirin quantification in combination treatment of chronic hepatitis C. *Antimicrob. Agents Chemother.* 2003; **47**: 124–9.

42 Poynard T, McHutchison J, Goodman Z, Ling MH, Albrecht J. Is an 'a la carte' combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? The ALGOVIRC Project Group. *Hepatology* 2000; **31**: 211–18.

43 Seeff LB, Hoofnagle JH. National Institutes of Health Consensus Development Conference: management of hepatitis C 2002. *Hepatology* 2002; **36**: S1–2.

44 Hung CH, Lee CM, Lu SN *et al.* Mutations in the NS5A and E2-PePHD region of hepatitis C virus type 1b and correlation with the response to combination therapy with interferon and ribavirin. *J. Viral Hepat.* 2003; **10**: 87–94.

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Mutagenic effects of ribavirin and response to interferon/ribavirin combination therapy in chronic hepatitis C☆

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See Editorial, pages 553–555

***Background/Aims*: To elucidate whether ribavirin acts as a mutagen in the clinical setting and to clarify the relationship between ribavirin-induced mutations and virological response to combined therapy.**

***Methods*: Thirty-four patients with hepatitis C virus (HCV) genotype 1b received ribavirin monotherapy for 4 weeks, followed by a 24-week course of IFN/ribavirin therapy. HCV mutations during a non-treatment observation period and during subsequent ribavirin monotherapy were determined, and the relationship between mutations and response to subsequent IFN/ribavirin therapy was evaluated.**

***Results*: Serum HCV significantly decreased from 6.90 to 6.56 $\log_{10}$copy/ml in response to ribavirin monotherapy ($P<0.0001$). Nucleotide mutations in the NS5A and NS5B regions occurred during ribavirin monotherapy at a rate of 2.9×10^{-2}/site/year and 1.3×10^{-2}/site/year, respectively, a significantly higher rate than the mutation rates during the prior non-treatment observation period (0.60×10^{-2}/site/year and 0.24×10^{-2}/site/year, $P=0.02$, respectively). Mutation rates in the NS5A region were significantly higher in sustained viral responders (SVRs, $n=10$) than in non-responders (8.8×10^{-2}/site/year vs. 0.38×10^{-2}/site/year, $P=0.0005$, respectively). In the NS5A region, non-synonymous mutations only occurred in SVRs.**

***Conclusions*: Ribavirin may act as a mutagen, and mutations occurring during ribavirin therapy correlate with the virological response to subsequent IFN/ribavirin combination therapy.**

Keywords: NS5A; NS5B; ISDR; HCV; HCV dynamics

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☆ The authors who have taken part in this study declared that they have not a relationship with the manufacturers of the drugs involved either in the past or present and did not receive funding from the manufacturers to carry out their research. The nucleotide sequences reported in this paper will appear in the DDBJ/EMBL/GenBank with accession numbers AB207766 through AB207801.

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; RdRp, RNA dependent RNA polymerase; PCR, polymerase chain reaction; SVR, sustained viral responder; NR, non-responder; ISDR, interferon sensitivity determining region.

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1. Introduction

Ribavirin, a synthetic guanosine analog, has broad antiviral effects against both DNA and RNA viruses. Although ribavirin monotherapy has minimal efficacy on hepatitis C viral (HCV) eradication [1–3], studies have reported higher sustained response rates following combination therapy with interferon (IFN)-alfa and ribavirin than following IFN-alfa monotherapy [4–7]. Several mechanisms of action of ribavirin have been proposed [8]. In vitro and animal studies, in particular, have demonstrated that the antiviral activity of ribavirin is exerted through its potent mutagenic effects on RNA viruses after being incorporated into newly synthesized genomes by viral RNA-dependent RNA polymerase (RdRp) [9–11]. Still, little information is available regarding the mechanisms responsible for the increased virological efficacy associated with concurrent administration of ribavirin and IFN. No clinical studies to date have determined whether ribavirin induces mutations in the clinical setting nor examined the relationship between the mutagenic effects of ribavirin and viral response to IFN/ribavirin combination therapy.

The present study evaluated a set of patients with chronic hepatitis C. To elucidate whether ribavirin acts as a mutagen in the clinical setting, for each subject the sequential nucleotide mutations occurring during ribavirin monotherapy were compared with mutations occurring in the same patient during the non-treatment observation period immediately preceding the initiation of ribavirin monotherapy as a control. The relationship between mutations observed during ribavirin monotherapy and viral response to subsequent IFN/ribavirin combination therapy was also determined.

2. Methods

2.1. Patients

Among patients with biopsy-proven chronic hepatitis C hospitalized at the Musashino Red Cross Hospital from December 2001 to June 2002, 34 patients of HCV genotype 1b with a high viral load (> 100 kcopies/ml by Amplicor-HCV monitor assay; Roche Molecular Diag. Co., Tokyo, Japan) were included in the present study (Table 1). Patients with liver cirrhosis, autoimmune hepatitis, and alcoholic liver injury were excluded from the study. No patient was positive for hepatitis B virus-associated antigen/antibody or anti-human immunodeficiency virus antibody. No patient received immunomodulatory therapy before enrolment in the study. Written informed consent was obtained from all patients, and this study was approved by the ethical committee of Musashino Red Cross Hospital in accordance with the Helsinki Declaration.

2.2. Treatment protocol and study design

Treatment schedule and time points for sequential genetic analysis are described in the upper part of Fig. 1. Following a non-treatment observation period, all patients received oral ribavirin daily for 4 weeks. Ribavirin dosage was 600 mg daily for patients who weighed less than 60 kg and 800 mg daily for patients who weighed between 60 and 80 kg. All patients subsequently received a 24-week course of treatment consisting of intramuscular IFN-alfa 2b (Intron, Schering-Plough, Kenilworth, NJ) at an initial dosage of 6 MU daily in combination plus daily oral ribavirin at the same dosage (600 mg daily or 800 mg daily) as was given during pretreatment monotherapy. After the first 2 weeks of IFN/ribavirin combination therapy, the IFN dosing frequency was reduced to 6 MU three times a week for the remaining 22 weeks.

Table 1
Clinical characteristics of the study patients

No. of patients	34
Age (years)	59±8
Gender (M/F)	14/20
Liver histology	
A1/A2/A3	14/15/5
F1/F2/F3	10/15/9
Number of ISDR mutations (0/1)	22/12
Baseline data	
ALT (IU/L)	81±56
Platelet count ($\times 10^3$/ml)	150±52
Viral load (KIU/ml)	572±204
SVR (%)	29.4 (10/34)

Values are expressed as mean±standard deviation.

Nucleotide sequences of the NS5A and NS5B regions of the HCV genome were determined at the following time points: (1) enrolment into the study; (2) end of the non-treatment observation period (immediately before initiation of ribavirin monotherapy); (3) end of the 4-week ribavirin monotherapy (immediately before initiation of IFN/ribavirin combination therapy). For each patient, nucleotide changes between time points 1 and 2 during the non-treatment observation period (mean: 6 months, range: 2–48 months) were used as a control to determine whether mutations observed during the period of ribavirin monotherapy (between time points 2 and 3) represented true effects of ribavirin. Mutation rates for the non-treatment observation period were compared with the mutation rates during the subsequent ribavirin monotherapy period. Moreover, the relationships between observed mutations and the clinical outcome of the subsequent IFN/ribavirin therapy were evaluated.

2.3. Nucleotide sequencing

Nucleotide sequences of the NS5A and NS5B regions of the HCV genome were determined by direct sequencing of polymerase chain reaction (PCR)-amplified DNA, as previously described [12,13]. In brief, after RNA was extracted from sera of the study subjects, NS5A and NS5B regions were amplified by RT-PCR using Taq polymerase, and the sequences corresponding to nucleotides 6703–7320 and 7730–8874 of HCV-J were determined [13]. The sequences of the primers for the NS5A region were the same as described previously [12]. Sequences for the NS5B region, which were amplified with two partially overlapping sets of primers, were as follows: NS5B1-5′ outer set, 5′GGTGAATACCTGGAAATCAAAGAAA3′; NS5B1-3′ outer set, 5′AGAAATGAGTCATCAGAATCATCCT3′; NS5B1-5′ inner set, 5′TGTAAAACGACGGCCAGTATGGGCTTCTCATATGACAC3′; NS5B1-3′ inner set, 5′CAGGAAACAGCTATGACCCATGATGATGTTGCCTAGCC3′; NS5B2-5′ outer set, 5′GCAGAAGAAGGTCACCTTTGACAGA3′; NS5B2-3′ outer set, 5′TCGGGGGCCAAGTCACAACATTGGT3′; NS5B2-5′ inner set, 5′TGTAAAACGACGGCCAGTTTTGACAGACTGCAAGTCCT3′; NS5B2-3′ inner set, 5′CAGG AAACAGCTATGACCTTCTCAGTGACCGTTGAGTC3′. M13-forward and M13-reverse sequences were attached to the 5′-termini of sense and anti-sense nested PCR primers. Both strands of the PCR products were cycle sequenced with the PRISM dye termination kit (CN402069, Applied Biosystems, Chiba, Japan), and nucleotide sequences were determined by an automated DNA sequencer Model 373A (Applied Biosystems).

Mutations resulting from ribavirin therapy were defined as the detection of a new nucleotide which was not detected as even a minority population in the prior specimen from that subject. Electropherograms were read by two independent readers without knowledge of the patients' backgrounds and outcomes. A nucleotide detected in the post-ribavirin monotherapy specimen was considered to be a 'new nucleotide' only when both readers could not identify any tracking peak of this nucleotide in a prior specimen.

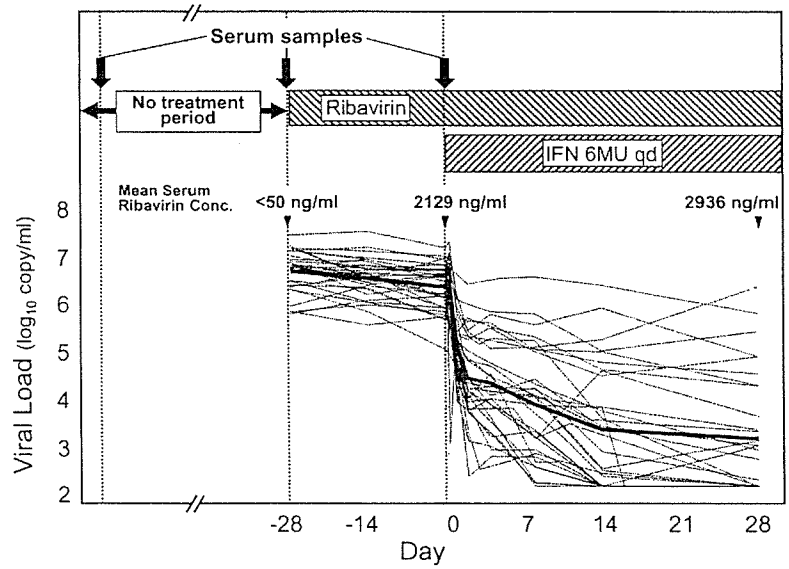

Fig. 1. Treatment schedule and HCV dynamics during ribavirin monotherapy and subsequent IFN/ribavirin combination therapy. After a non-treatment observation period, all patients received oral ribavirin daily for 4 weeks and subsequently received intramuscular IFN-alpha 2b in combination with daily oral ribavirin. For the first 2 weeks of IFN/ribavirin combination therapy, 6 MU of IFN-alpha 2b was given daily; the IFN dosing frequency was then reduced to 6 MU three times a week for the remaining 22 weeks of combination therapy. Serum HCV dynamics of individual patients are shown in dotted lines, and the solid line represents the mean of these values. The nucleotide sequences were serially analyzed at the time points indicated by the arrows. Closed triangles indicate the time points of serum ribavirin concentration measurements.

Peaks less than 10% of the dominant peak were considered to be background signals. To calculate values for *dN* (nonsynonymous substitution), *dS* (synonymous substitution), *dN/dS* ratios, and the number of point mutations, analyses of nucleotide sequences were performed using a software program (MEGA version 2.1.). Separate *dN/dS* ratios were determined for each NS5 region in patients who had nucleotide mutations in the corresponding NS5 regions. To determine the locations of amino acid mutations in the tertiary structure of the NS5B molecule, a crystal structure model of HCV NS5B-RdRp was constructed using 1QUV from the Protein Data Bank. A space-filling representation of each atom was generated using Rasmol 2.7.2.1. The deduced amino acid sequences of the NS5A and NS5B regions were also compared with a prototype HCV 1b strain, HCV-J [14].

2.4. HCV dynamics in serum

To analyze the effect of ribavirin on viral dynamics, HCV-RNA concentrations were quantified just before and at the end of ribavirin monotherapy, and also at 4, 8, 24, 48, 96, 192, and 336 hours after initiating IFN/ribavirin combination therapy, using real-time detection PCR, as reported previously [15–17]. The detection sensitivity of this assay is approximately 10 copies/ml, and the dynamic range is from 10 copies/ml to more than 1×10^8 copies/ml [17]. For each patient, the viral decline curve was plotted on a semilogarithmic scale, and the slopes of the exponential viral declines were calculated for each viral decline phase by a straight-line fit of the data.

2.5. Definitions of response to therapy

A patient negative for serum HCV-RNA during the first six months following the completion of IFN/ribavirin combination therapy was defined as a sustained viral responder (SVR), and a patient positive for HCV-RNA during this time period was defined as a non-responder (NR).

2.6. Statistical analysis

Categorical data were compared by the chi-square test or Fisher's exact test. Distributions of continuous variables were analyzed by the Student's *t*-test for two groups. All tests of significance were two-tailed, and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. HCV dynamics

During the 4-week period of ribavirin monotherapy, the mean serum HCV-RNA level significantly decreased from 6.90 to 6.56 log_{10} copy/ml ($P<0.0001$, paired t test) (Fig. 1). Serum HCV dynamics after the start of subsequent IFN/ribavirin combination therapy demonstrated a biphasic kinetic pattern of HCV-RNA decline. The exponential decay slopes for the first phase and the second phase were 2.00 ± 0.77 log_{10}/day and 0.15 ± 0.14 log_{10}/day, respectively.

3.2. The effect of ribavirin on HCV gene mutation and the relationship between mutations and virological response to IFN/ribavirin combination therapy

In a pairwise comparison of the NS5 sequences before and after ribavirin monotherapy, new HCV gene mutations occurring during ribavirin monotherapy were observed in the NS5A region in10 patients and in the NS5B region in 8 patients. Mean gene mutation rates in the NS5A and NS5B regions during ribavirin administration were 2.9×10^{-2}/site/year and 1.3×10^{-2}/site/year, respectively, rates which were significantly higher compared with the rates observed during the prior non-treatment observation periods in

Table 2
Mutation rates during the non-treatment observation period and during subsequent ribavirin monotherapy

	NS5A	NS5B
During non-treatment observation period		
Total	0.6×10^{-2}	0.24×10^{-2}
SVR	1.4×10^{-2} ‡	0.46×10^{-2}
NR	0.28×10^{-2} ‡ *	0.14×10^{-2} †
During subsequent ribavirin monotherapy		
Total	2.9×10^{-2}	1.3×10^{-2}
SVR	8.8×10^{-2} §	2.2×10^{-2}
NR	0.38×10^{-2} §	0.96×10^{-2}

Note that the difference in NS5A mutation rate between SVR and NR patients was greater during ribavirin treatment than that during the non-treatment observation (control) period (23-fold vs. 5-fold). *$P=0.02$ (paired t test). †$P=0.02$ (paired t test). ‡$P=0.04$ (unpaired t test). §$P=0.0005$ (unpaired t test).

the same patients (Table 2, $P=0.02$ for both NS5 regions). Of all nucleotide mutations, 72.1% in the NS5A region and 85.5% in the NS5B region were transition mutations. Percentages of transition mutations for all mutations are detailed in Table 3. Mutations from C to T and from G to A were frequent (Table 3). Synonymous mutations occurred more frequently than non-synonymous mutations in both regions, comprising 80.6% of all NS5A mutations and 73.5% of all NS5B mutations. No significant correlation was found between gene mutation rates and the subject's serum ribavirin concentration at the end of four weeks of ribavirin monotherapy.

Next, the relationship between mutations during ribavirin therapy and virological response to combination therapy was evaluated. The sustained viral response rate was 24.9% (10/34) (Table 1). The proportion of patients who had mutations in the NS5A region during ribavirin monotherapy was significantly higher in SVRs than in NRs (8 out of 10 vs. 2 out of 24 patients, respectively, $P<0.0001$, Fisher's exact test). Correspondingly, gene mutation rates in the NS5A region were significantly higher in SVRs than in NRs: 8.8×10^{-2}/site/year vs. 0.38×10^{-2}/site/year, respectively ($P=0.0005$) (Table 2). In the NS5B region, although statistically significant differences were not observed, the gene mutation rate tended to be higher in SVRs than in NRs: 2.2×10^{-2}/site/year vs. 0.96×10^{-2}/site/year, respectively (Table 2). The proportion of patients with mutations in the NS5B region did not significantly differ between SVRs and NRs.

Table 3
Transition mutations during the non-treatment observation period and during subsequent ribavirin monotherapy

	C to T	T to C	G to A	A to G	Others[a]
During non-treatment observation period					
NS5A (%)	22.2	33.3	22.2	11.1	11.2
NS5B (%)	28.5	57.1	0	14.3	0
During subsequent ribavirin monotherapy					
NS5A (%)	18.6	9.3	25.6	18.6	27.9
NS5B (%)	38.5	20.5	10.2	16.3	14.5

Data are expressed as a percentage of all mutations observed in each region.
[a] Transversion mutations were included in this column.

3.3. Non-synonymous mutations and virological response to IFN/ribavirin combination therapy

In the NS5A region, non-synonymous mutations were found in 19.4% of all nucleotide mutations observed during ribavirin monotherapy. Alterations in deduced amino acid residues by non-synonymous mutations are illustrated in Fig. 2, which shows all 10 patients who had nucleotide mutations during ribavirin monotherapy. In a pairwise comparison between pre- and post-ribavirin monotherapy, amino acid alterations were found in 5 of these 10 patients. Non-synonymous mutations were found exclusively in SVRs. In other words, 5 out of the 8 patients who achieved SVR status had 1 or 3 non-synonymous mutations; 2 of these 5 patients had amino acid alterations accompanied with an increase in the number of amino acid mutations in the interferon sensitivity determining region (ISDR). In contrast, all nucleotide mutations detected in NRs were synonymous mutations. dN/dS tended to be higher in SVRs than in NRs ($P=0.25$, Fig. 3).

In the NS5B region, non-synonymous mutations were detected in 3 out of the 10 SVRs and in 2 out of the 24 NRs. The proportion of patients with non-synonymous mutations did not significantly differ between the two response groups. dN/dS also did not significantly differ between the SVRs and the NRs ($P=0.77$, Fig. 3). Of the 9 amino acid mutations detected in the NS5B region, two were located in the functional domain of the RdRp, one was located in domain C (D310N), and one was located in domain D (R345S). As shown by the crystal model of the NS5B-RdRp in Fig. 4, amino acid mutations were primarily located on the molecular surface; none occurred in the nucleotide groove or in the tunnel.

3.4. Genetic changes during the non-treatment observation period

Gene mutation rates in the NS5A and NS5B regions during the non-treatment observation period were calculated as 0.60×10^{-2}/site/year and 0.24×10^{-2}/site/year, respectively, rates which were significantly lower than the mutation rates observed during ribavirin monotherapy (Table 2). Next, the relationships between viral mutation rates during

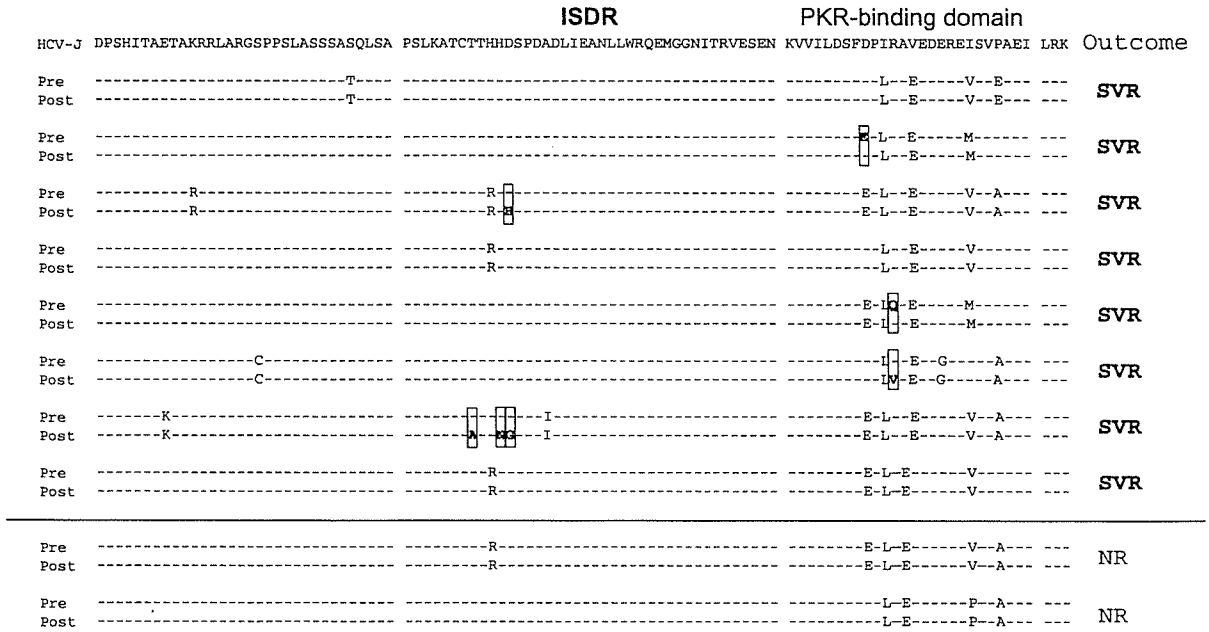

Fig. 2. Amino acid sequence alignments in the NS5A region obtained pre- and post-ribavirin monotherapy. The figure contains data for all 10 patients out of 34 with nucleotide mutations occurring during ribavirin monotherapy. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate identical amino acid residues, with the consensus sequence and HCV-J shown at the top. Outcomes of combination therapy are shown on the right side. In a pairwise comparison between pre- and post-ribavirin monotherapy sera, amino acid alterations were found in 5 of 10 patients. Non-synonymous mutations were exclusively found in SVR patients.

the non-treatment observation period and viral responses to subsequent IFN/ribavirin therapy were evaluated. Interestingly, gene mutation rates in the NS5A region during the non-treatment observation period were significantly higher in SVRs than in NRs: 1.4×10^{-2}/site/year vs. 0.28×10^{-2}/site/year, respectively ($P=0.04$). However, it should be noted that the relative difference in NS5A mutation rates between SVRs and NRs during ribavirin treatment was larger than the relative difference between SVRs and NRs during the non-treatment observation period (23-fold vs. 5-fold, $P=0.01$).

Similarly, gene mutation rates in the NS5B region were higher in SVRs (0.46×10^{-2}/site/year) than in NRs (0.14×10^{-2}/site/year), although these differences were not statistically significant. Seven of the nine patients who had mutations occurring during the non-treatment observation period also had mutations during ribavirin monotherapy. However, 53% (8/15) of the patients with mutations during ribavirin monotherapy had no gene mutations during the non-treatment observation period.

4. Discussion

In the present study, we identified HCV gene mutations occurring during ribavirin monotherapy and found that the mutation rate was associated with the virological response to subsequent IFN/ribavirin combination therapy. Since the mutation rate was significantly higher during ribavirin monotherapy than during non-treatment observation periods in the same patients, at least some of the mutations observed during ribavirin treatment were likely an effect of ribavirin administration. Therefore, ribavirin appears to act as a mutagen during clinical treatment, and this mutagenic effect correlates with improvements in the virological response rate resulting from the synergistic use of ribavirin with IFN.

Recently, several in vitro and animal studies [9–11, 18,19] have provided evidence that ribavirin has mutagenic

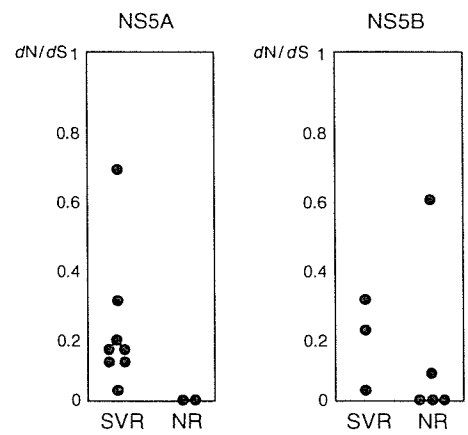

Fig. 3. Ratio of *dN* (nonsynonymous substitution) to *dS* (synonymous substitution) distances for the NS5A and NS5B regions. All 10 patients with nucleotide mutations in the NS5A region and all 8 patients with nucleotide mutations in the NS5B region during ribavirin monotherapy are shown in this figure. All pairwise *dN/dS* ratios were calculated using MEGA ver. 2.1 for each subject. dN/dS in the NS5A region tended to be higher in SVR patients than in NR patients (NS5A: $P=0.25$, NS5B: $P=0.77$).

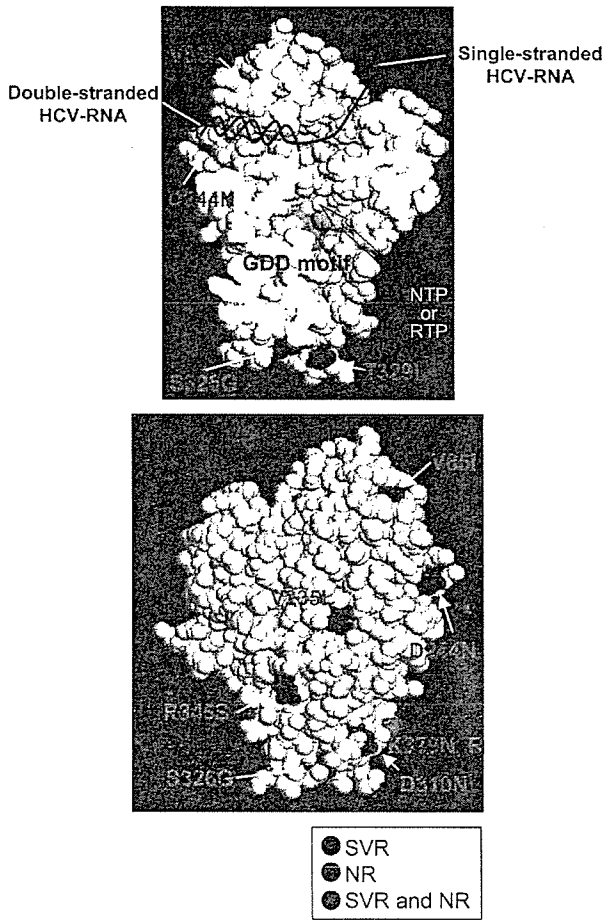

Fig. 4. Crystal structure of HCV NS5B-RNA dependent RNA polymerase. The molecular model of NS5B was constructed using 1QUV from the Protein Data Bank. A space-filling representation of each atom is shown. Graphics were generated using Rasmol 2.7.2.1. View of a cross section of the RdRp at level of nucleotide tunnels (A) and the surface (B) are illustrated. Single stranded HCV RNA enters the enzyme through a groove at the top of the finger domain, and NTP (or ribavirin triphosphate; RTP) enters the enzyme through the right lower NTP tunnel (between the fingers and the thumb). The substitutions observed in SVR patients only, NR patients only, and both sets of patients are shown in red, blue, and magenta, respectively.

activity. Other studies have found that ribavirin increases the mutation rate in a full-length HCV cDNA plasmid [20] and in an HCV replicon [21]. However, none of these studies were human studies; no study to date has documented that ribavirin has mutagenic activity in the clinical setting. In the absence of clinical data, the association between the mutagenic activity of ribavirin and the clinical and virological responses to IFN/ribavirin combination therapy remains unknown. Detecting gene mutations induced by ribavirin and analyzing their association with clinical responses to IFN/ribavirin therapy are extremely difficult because HCV with error mutations may be immediately eliminated by the concurrently administered IFN. Although issues regarding the mutagenic effects of ribavirin remain controversial, the inclusion of four weeks of ribavirin monotherapy immediately before IFN/ribavirin combination therapy in our protocol enabled us to clarify the association between gene mutations induced by ribavirin and the ensuing virological response to the subsequent IFN/ribavirin therapy.

Interestingly, the correlation between mutation rate and virological response to therapy was more evident in the NS5A region, including the ISDR, than in the NS5B region. From our previous analysis of the full-length HCV genome [13,22], NS5A sequences were more variable than NS5B sequences among different clones. Therefore, it seems likely that the stronger relationship observed between mutation rates in the NS5A region and SVR status was due to the relatively greater inherent variability of the NS5A region compared with the NS5B region. Even in the NS5A region, however, serial amino acid mutations rarely occur in the same patients during untreated periods [23]. Hence, our ribavirin monotherapy results suggest that in some patients, ribavirin induces non-synonymous mutations which increase sensitivity to IFN. In turn, this increased susceptibility to IFN could lead to SVR status. Conversely, no functionally important mutations were detected in the NS5B region. Since a ribavirin-induced non-synonymous mutation at a functionally important site in the NS5B region is likely to lead to viral death, even without concurrent IFN administration, the substitutions observed in this critical region are more probably the results of positive or negative selection.

The patients who had gene mutations during the non-treatment observation period were prone to also having mutations during ribavirin monotherapy as well and were more likely to achieve SVR status. These correlations suggest that ribavirin easily induced HCV mutations in such patients. Although mutations could have occurred in the absence of ribavirin, the difference in mutation rates between SVRs and NRs was significantly larger during ribavirin treatment than during the non-treatment observation period (23-fold vs. 5-fold, $P=0.01$). The observation that in more than half of the patients, mutations occurred only during ribavirin monotherapy and were not detectable during the non-treatment observation period suggests that mutagenic effects of ribavirin synergistically potentiating the virological response to IFN may play an important role in achieving SVR status.

Gene mutations in the NS5A and/or NS5B regions during ribavirin monotherapy did not occur in all patients in the present study, suggesting that the intensity of the mutagenic effects of ribavirin differed among individual patients. Additionally, some patients who did not have gene mutations in these regions during the non-treatment observation period or during ribavirin monotherapy nonetheless still achieved SVR, suggesting that the synergistic efficacy of ribavirin may not result solely from the mutagenic activity of this agent. Alternatively, ribavirin may have possibly induced mutations during the period of IFN/ribavirin combination therapy in these patients, but as previously discussed, the concurrent IFN might have eliminated the HCV containing

these mutations before the mutations were able to be detected. Additionally, our data did not address the significance of the impact that mutations in regions other than NS5 may have had on viral response to therapy.

HCV populations in vivo consist of a quasispecies nature. Our previous cloning analysis detected small number of minor clones in specimens, which were determined as ISDR-wild type by direct sequencing [24]. Hence, it should be noted that our criteria for mutation could not completely distinguish between de novo mutation and selection of a minor clone.

In the present study, we found that ribavirin also expressed antiviral activity by reducing viral load, presumably because we used a highly quantitative assay for HCV-RNA measurement [17]. However, contradictory results have been reported previously [1–3]. Since the present study identified only a small reduction in viral load, further investigation is needed to confirm our result.

In conclusion, our data demonstrate that clinical administration of ribavirin induces mutations in HCV genes and suggest that, in some patients, mutagenesis may be one of the mechanisms responsible for the synergistic efficacy of ribavirin in IFN/ribavirin combination therapy.

References

[1] Kakumu S, Yoshioka K, Wakita T, Ishikawa T, Takayanagi M, Higashi Y. A pilot study of ribavirin and interferon beta for the treatment of chronic hepatitis C. Gastroenterology 1993;105: 507–512.

[2] Reichard O, Andersson J, Schvarcz R, Weiland O. Ribavirin treatment for chronic hepatitis C. Lancet 1991;337:1058–1061.

[3] Dusheiko G, Main J, Thomas H, Reichard O, Lee C, Dhillon A, et al. Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled study. J Hepatol 1996;25:591–598.

[4] Reichard O, Norkrans G, Fryden A, Braconier JH, Sonnerborg A, Weiland O. Randomised, double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. The Swedish Study Group. Lancet 1998;351:83–87.

[5] Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, et al. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet 1998;352: 1426–1432.

[6] McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med 1998;339:1485–1492.

[7] Davis GL, Esteban MR, Rustgi V, Hoefs J, Gordon SC, Trepo C, et al. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. N Engl J Med 1998;339:1493–1499.

[8] Lau JY, Tam RC, Liang TJ, Hong Z. Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. Hepatology 2002;35:1002–1009.

[9] Crotty S, Maag D, Arnold JJ, Zhong W, Lau JY, Hong Z, et al. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat Med 2000;6:1375–1379.

[10] Crotty S, Cameron CE, Andino R. RNA virus error catastrophe: direct molecular test by using ribavirin. Proc Natl Acad Sci USA 2001;98: 6895–6900.

[11] Lanford RE, Chavez D, Guerra B, Lau JY, Hong Z, Brasky KM, et al. Ribavirin induces error-prone replication of GB Virus B in primary Tamarin hepatocytes. J Virol 2001;75:8074–8081.

[12] Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996;334:77–81.

[13] Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest 1995;96:224–230.

[14] Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 1990;87:9524–9528.

[15] Asahina Y, Izumi N, Uchihara M, Noguchi O, Tsuchiya K, Hamano K, et al. A potent antiviral effect on hepatitis C viral dynamics in serum and peripheral blood mononuclear cells during combination therapy with high-dose daily interferon alfa plus ribavirin and intravenous twice-daily treatment with interferon beta. Hepatology 2001;34:377–384.

[16] Asahina Y, Izumi N, Uchihara M, Noguchi O, Nishimura Y, Inoue K, et al. Interferon-stimulated gene expression and hepatitis C viral dynamics during different interferon regimens. J Hepatol 2003;39: 421–427.

[17] Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636–642.

[18] Maag D, Castro C, Hong Z, Cameron CE. Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. J Biol Chem 2001;276:46094–46098.

[19] Vo NV, Young KC, Lai MM. Mutagenic and inhibitory effects of ribavirin on hepatitis C virus RNA polymerase. Biochemistry 2003; 42:10462–10471.

[20] Contreras AM, Hiasa Y, He W, Terella A, Schmidt EV, Chung RT. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. J Virol 2002;76: 8505–8517.

[21] Tanabe Y, Sakamoto N, Enomoto N, Kurosaki M, Ueda E, Maekawa S, et al. Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. J Infect Dis 2004;189:1129–1139.

[22] Hamano K, Sakamoto N, Enomoto N, Izumi N, Asahina Y, Kurosaki Y, et al. Mutations in the NS 5B region of the hepatitis C virus genome correlate with clinical outcomes of interferon-alpha plus ribavirin combination therapy. J Gastroenterol Hepatol; in press.

[23] Maekawa S, Enomoto N, Kurosaki M, Nagayama K, Marumo F, Sato C. Genetic changes in the interferon sensitivity determining region of hepatitis C virus during the natural course of chronic hepatitis C. J Med Virol 2000;61:303–310.

[24] Sakuma I, Enomoto N, Kurosaki M, Izumi N, Marumo F, Sato C. Differential effect of interferon on hepatitis C virus 1b quasispecies in the nonstructural protein 5A gene. J Infect Dis 1999;180: 1001–1009.

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Polymerase Domain B Mutation Is Associated with Hepatitis Relapse during Long-Term Lamivudine Therapy for Chronic Hepatitis B

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Key Words
Lamivudine · Breakthrough hepatitis · INNO-LiPA · Hepatitis B virus DNA · Domain B

Abstract
Breakthrough hepatitis remains the major issue in long-term lamivudine therapy for chronic hepatitis B. However, the emergence of drug-resistant hepatitis B virus (HBV) is not always accompanied by a relapse of hepatitis. To elucidate factors predictive of breakthrough hepatitis, 53 patients with genotype C of HBV on long-term lamivudine therapy were analyzed. HBV reappeared during therapy in 19 patients with a cumulative incidence of 15% at 1 year, 34% at 2 years, and 60% at 3 years. Within this group, breakthrough hepatitis developed in 12 patients (63%). A polymerase gene domain B mutation (rt180M) emerged in 13 patients, and domain C mutations (rt204I, rt204V) were found in 19 patients. The rt180M mutation was associated with breakthrough hepatitis ($p < 0.05$) with a positive predictive value of 85% and a negative predictive value of 83%. Patients with the rt180M mutation had higher HBV-DNA levels during viral breakthrough compared to patients with rt180wt ($p < 0.05$). The mutational pattern of rt204 was not associated with breakthrough hepatitis. In conclusion, genotypic assays for the rt180M mutation after viral breakthrough may be useful in predicting the risk of breakthrough hepatitis and in deciding when to initiate alternative or additive nucleoside analogue therapy.

Introduction

Lamivudine, a nucleoside analogue, is now widely used as primary therapy for chronic hepatitis B virus (HBV) infection [1]. The initial clinical response is usually favorable with high rates of HBV suppression, normalization of serum alanine transaminase (ALT) levels, loss of detectable serum HBe antigen, as well as histologic improvement [2–7]. However, short-term therapy cannot completely eliminate the HBV pool in the liver [8], and cessation of therapy usually leads to withdrawal hepatitis [9–11]. Consequently, long-term therapy is required in the majority of patients to maintain the suppressive effects of lamivudine.

Unfortunately, long-term therapy has drawbacks as well. Response rates may gradually decrease due to the emergence of drug-resistant HBV. This resistant virus form is characterized by amino acid mutations in the catalytic domains of the polymerase gene. Two mutations are frequently observed in association with lamivudine resistance: a mutation of methionine to isoleucine or va-

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line in codon 204 of the catalytic domain C, and a mutation of leucine to methionine in codon 180 of the catalytic domain B [12]. The emergence of these mutations leads to the reappearance of HBV-DNA (viral breakthrough) and the relapse of hepatitis (breakthrough hepatitis) [13] at cumulative rates of 14–32% at 1 year and 38–49% at 2 years [2, 4, 5, 14, 15].

However, biochemical changes do not always correlate with drug resistance. In some cases of viral breakthrough during long-term lamivudine therapy, patient ALT levels remain unaffected, and these patients continue to exhibit histologic improvement [12, 15]. Conversely, there are also patients who develop a severe flare-up of their hepatitis or even frank decompensation when viral resistance emerges [6, 16–20]. The risk of hepatitis B relapse thus becomes an important issue in patients undergoing long-term treatment with lamivudine. While various factors such as pretreatment HBV-DNA levels, ALT levels, presence of HBe antigen, and certain genotypes, may be associated with the emergence of resistant viral strains, factors predictive of a patient's clinical outcome have not yet been defined. Given the wide variability in patient responses to viral breakthrough and the potential morbidity and mortality associated with the worst outcomes, identifying pretreatment factors predictive of breakthrough hepatitis could be very relevant to clinical practice.

Finally, when analyzing treatment resistance, it is also important to consider the HBV viral genotype. Viral genotype may have some bearing on patient outcomes with long-term lamivudine therapy. For example, patients infected with genotype A who develop viral breakthrough while on lamivudine tend to have higher HBV-DNA levels than patients who harbor other HBV genotypes such as D. Investigators have recently shown that the pattern of polymerase gene mutation leading to lamivudine resistance may be different for genotype A [21]. A double mutation of methionine to valine in codon 204 and leucine to methionine in codon 180 was prevalent in genotype A, while a methionine to isoleucine mutation in codon 204 occurred more frequently in genotype D. These differences in mutational patterns may be linked to an association between genotype and HBV-DNA levels after viral breakthrough.

Currently, the most prevalent HBV genotype in Japan is genotype C [22]. Genotype C is reported to be associated with a more aggressive clinical course and increased resistance to interferon therapy when compared to genotype B [23–25]. Given the pertinence of genotype in lamivudine resistance, differentiating the clinical significance of the various polymerase gene mutations in genotype C becomes critical.

The aim of the present study is to elucidate factors associated with breakthrough hepatitis during long-term lamivudine therapy in HBV genotype C infections. Mutations in domains B and C of the polymerase gene, core promoter gene and precore gene were analyzed to determine if specific mutational patterns might be associated with different clinical outcomes.

Patients and Methods

Therapeutic Protocol

Fifty-three patients with chronic hepatitis B genotype C who were consecutively started on long-term lamivudine monotherapy between August 1999 and November 2003 at Musashino Red Cross Hospital were analyzed retrospectively. There were 32 males and 21 females; mean age was 48.8 ± 11.8 years. At the start of therapy, all patients had detectable levels of HBV-DNA in their blood by polymerase chain reaction (PCR), as well as elevations in serum ALT levels. All patients were also found not to have either hepatitis C or human immunodeficiency virus antibodies in their blood. No patient received interferon or any other antiviral agents during the study or within 6 months of initiating lamivudine therapy. Patients were treated with a single oral dose of 100 mg of lamivudine every day; median duration of lamivudine therapy was 689 (range 207–1,736) days. All patients remained on lamivudine therapy throughout the course of study except those who developed breakthrough hepatitis. Informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Quantification of HBV-DNA

Blood samples were obtained at the start of therapy and then once every month during therapy. The serum level of HBV-DNA was determined using transcription-mediated amplification and hybridization protection assays (Fujirebio Inc., Tokyo, Japan) that have a detection range of 3.7–8.7 log genome equivalents (LGE)/ml [26].

Definitions

Viral breakthrough was defined as an elevation of more than 1 LGE/ml of HBV-DNA accompanied by mutations in the polymerase gene on 3 consecutive determinations during monthly testing after a period of HBV-DNA suppression. No case that met this definition experienced a spontaneous decline in HBV-DNA thereafter. Breakthrough hepatitis was defined as a sustained elevation in serum ALT levels on 2 consecutive determinations 2 weeks apart in concert with viral breakthrough.

Analysis of Precore and Core Promoter Mutations

Mutations in the precore and core promoter regions were analyzed at baseline. The A1762T and G1764A mutations in the basic core promoter [27] were detected by a commercially available en-

zyme-linked specific probe assay (Smitest HBV core promoter mutation detection kit, Genome Science Laboratory, Tokyo). The G1896A stop codon mutation in the precore region was detected by an enzyme-linked mini sequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostics, Tokyo). Use of both of these assays has been described previously [28].

Analysis of Lamivudine-Resistant HBV

Blood samples at the time of viral breakthrough were analyzed for mutations in the HBV polymerase associated with lamivudine resistance using INNO-LiPA HBV DR analysis (Innogenetics, Inc., Ghent, Belgium) [29, 30]. Briefly, DNA isolated from the serum was amplified by nested PCR and used for hybridization to the LiPA strips. The probes on the INNO-LiPA HBV DR strip cover the amino acids of codon 180 (wild-type leucine (L) and mutant methionine (M)) and codon 204 (wild-type methionine and mutants valine (V) and isoleucine (I)). The amino acid positions on the HBV polymerase gene are numbered for consistency with the newly established standardization of nomenclature for lamivudine-resistance mutations rt180M and rt204V/I (originally designated as L528M or L526M and M552V/I or M550V/I) [31].

Statistical Analysis

For statistical analysis the STAT View software package was used. Categorical data were analyzed using Fisher's exact test. Continuous variables were compared with Student's t test. A Kaplan-Meier estimate and the log-rank test were used to calculate the median time to and the significance of viral breakthrough, as well as the median time to and the significance of breakthrough hepatitis. Cox's proportional hazard model and stepwise logistic regression analysis were used for multivariate analysis. A p value of <0.05 was considered statistically significant.

Results

Patient Characteristics, Pretreatment Variables and Clinical Course

Prior to initiation of lamivudine therapy, the mean HBV-DNA level was 7.1 ± 1.1 LGE/ml, and the mean ALT was 215 ± 285 U/l. Of a total of 53 patients infected with genotype C of HBV, 30 patients had detectable HBeAg in their serum (56.6%). A precore stop codon mutation was detected in 21 patients (42%), and core promoter mutations were detected in 44 patients (88%). The median treatment period was 689 (range 207–1,736) days.

During therapy, detectable levels of HBV-DNA fell below 4 LGE/ml in 42 (79.3%) patients. Viral breakthrough occurred in a total of 19 patients; in these patients, the median time to viral breakthrough was 473 (range 224–1,128) days. The cumulative incidence of viral breakthrough was 15% at 1 year, 34% at 2 years, and 60% at 3 years (fig. 1).

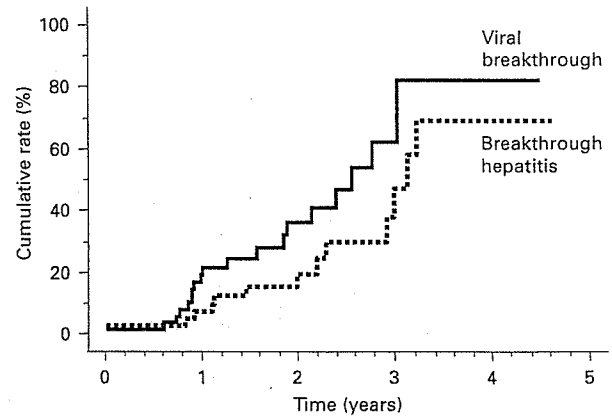

Fig. 1. The cumulative rate of viral breakthrough and breakthrough hepatitis. Kaplan-Meier plot of time to viral breakthrough and time to breakthrough hepatitis in 53 patients treated with lamivudine.

Among those 19 patients who developed viral breakthrough, 12 patients (63%) also developed breakthrough hepatitis. The median time to hepatitis after viral breakthrough was 111 days. The other 7 patients remained in biochemical remission. The cumulative incidence of breakthrough hepatitis was 4% at 1 year, 17% at 2 years, and 45% at 3 years (fig. 1).

Pretreatment variables including age, gender, presence of HBe antigen, HBV-DNA levels, ALT levels, and precore and core promoter mutations were analyzed. These variables were not found to be associated with viral breakthrough or with breakthrough hepatitis (table 1).

Variables at 24 Weeks of Treatment

HBV DNA levels after 24 weeks of lamivudine therapy correlated significantly with eventual viral breakthrough. At week 24 of therapy, HBV-DNA levels were above 4 LGE/ml in 23 patients. Moreover, there was a significant difference in time to viral breakthrough between those whose HBV-DNA levels were above and those whose levels were below 4 LGE/ml after 24 weeks of therapy ($p = 0.007$, Kaplan-Meier log-rank test; fig. 2a). Patients with HBV-DNA levels above 4 LGE/ml had a 3.5-fold higher probability of viral breakthrough compared to the other patients (Cox's proportional hazard model, 95% CI 1.33–9.34, $p = 0.012$). In contrast, the HBV-DNA levels above 4 LGE/ml at week 24 were not associated with breakthrough hepatitis (fig. 2b).